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Gross Muscular Activity and Temperature Regulation in the Restrained Rat.* (22509)

ROSCOE G. BARTLETT, JR.,† VERNON C. BOHR, GLENN L. FOSTER, MILTON A. MILLER, AND ROGER H. HELMENDACH.
Department of Physiology, College of Medical Evangelists, Loma Linda, Calif.

Several explanations have been offered for the production of restraint hypothermia(1-4). One which has received particular attention is the suggestion that lightly restrained animals become hypothermic because of restricted muscular activity(3,4). While studying restraint hypothermia it appeared that those animals which struggled more experienced a greater drop in body temperature. This paper reports an experiment in which an attempt was made to correlate quantitatively the degree of struggling with the body temperature drop of restrained rats maintained in the cold.

Methods and materials. Thirty-six healthy adult male Sprague-Dawley rats (225-275 g) were enclosed in individual loosely fitting restraining cages and placed in a cold room (0° C ± 1°) on pivoted platforms of a "ballistograph" so fashioned that movements of the animals were transmitted to rocker arms which vertically deflected writing levers. The frequency response was such that non-visible shivering would not have been recorded. No resonant effect which would give overshoot was observed. Gross shivering was not observed but would have been recorded as well as other gross movements. Records were obtained on a long paper kymograph with a paper speed of about 8 cm/hr. In order to quantitate the degree of struggling, the kymograph records were placed on a shadow box and the area within the struggling curves (*i.e.*, the curves drawn along the upper and lower borders of the kymograph records of the amount of struggling) was traced on 100% rag coordinate paper. These curves were then cut out and weighed on an analytical balance to the nearest mg. The weight of a known area of paper was found, and the weight of the cut-out curves converted to sq. cm.

Body temperatures of animals were obtained with indwelling thermometers inserted in the rectum to read high colonic temperature at a depth of 6-8 cm. These indwelling thermometers should be considered a portion of the stressing technic.

Results. A typical record from the tests (Fig. 1) shows that there was a marked relationship between the degree of struggling and thermolability. That those animals which

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† Present address: Laboratory of Physical Biology, N.I.H., Bethesda, Md.

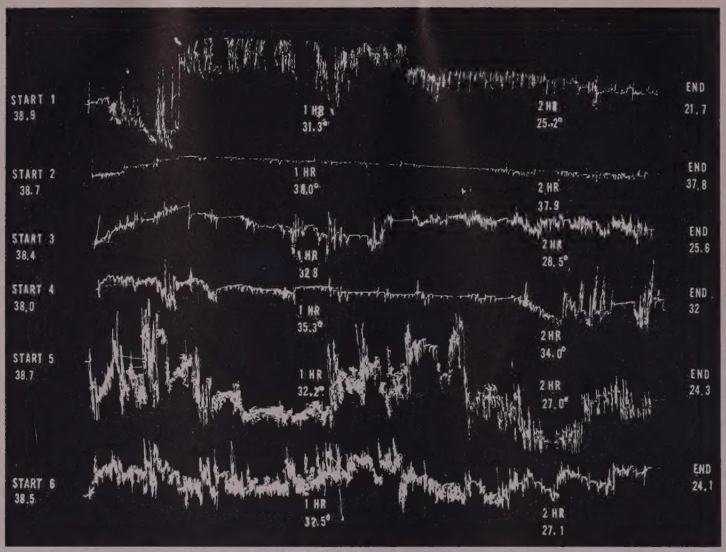

FIG. 1. Reproduction of a kymograph tracing from a typical test. Because the 6 records were obtained simultaneously, these 6 tracings were originally staggered. To improve the visual presentation, the tracings were separated and placed one above the other. The body temperature readings at the beginning, 1 hr, 2 hr, and at the termination of the tests are indicated on the records.

struggled more also became the more hypothermic is convincingly demonstrated in these records. The correlation coefficient between body temperature drop and degree of struggling was 0.56 ± 0.13, a significant level. When the fall in body temperature was plotted against the units of activity (both over a 2-hour period) for all animals a curve through the points rose to a plateau indicating that body temperature fall tended not to continue after the animals exceeded a certain activity level.

Discussion. The finding that marked and prolonged struggling seemed to limit the extent of body temperature drop is of interest. It appears that such struggling ultimately enables an animal to stabilize its temperature at a lower level. It is reasonable that if an animal's body temperature continues to fall it will ultimately restrict movement. It was observed that the lowering of body temperature did limit the amount of struggling in that an animal which struggled vigorously early in the test, thus experiencing a large drop in body temperature, struggled less later in the experiment.

It is not possible from the available data to determine a cause and effect relationship between struggling and the body temperature fall. It is likely that both struggling and thermolability are the effects of another cause. It has been demonstrated that those rats which are the more emotional become the more hypothermic when exposed to the stress of restraint in the cold(5). The relationship of struggling to emotionality is obvious, that of thermolability is not yet clear. It is known, however, that animals restrained in the cold consume more oxygen (and, therefore, presumably produce more heat) than do non-restrained, cold-exposed control animals(6). It may be, then, a matter of alteration of circulation within the skin which results in a greater net heat loss in spite of the increased heat production. Another possible relationship is that those animals which were rendered thermolabile by the stress of restraint experienced a drop in temperature which then initiated the struggling. If this were the sequence of events the struggling may actually have retarded the rate of body temperature fall. By inspection the records do not bear this out. It seems even less likely that the reverse relationship is true; *i.e.* struggling initi-

ated the body temperature fall.

The exact age of the animals was not known but their size (225-275 g) indicates that they were well over 60 days of age with well developed temperature regulating mechanisms(7).

The quantitative data presented here support our hypothesis that struggling and thermolability (hypothermia) are positively correlated in the restrained rat. We have no ready explanation for this apparent paradox. However, these data do negate the contention of some that body temperature drop in the restrained rat is a result of muscular inactivity.

Summary. Body temperature drop is positively correlated with gross body movement of rats restrained in the cold. It is concluded that restraint hypothermia cannot be explained on the basis of restricted muscular activity.

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Independence of Phosphatide Induced Hypercholesteremia and Hepatic Function.* (22510)

SANFORD O. BYERS AND MEYER FRIEDMAN.
(With technical assistance of Clarence Omoto and Warren Hayashi.)
Harold Brunn Institute, Mount Zion Hospital, San Francisco, Calif.

Previous studies(1-5) have suggested that the pathogenesis of various hypercholesteremias rested primarily on the retention and accumulation ("trapping") of cholesterol in plasma and plasma alone. Since phospholipid and/or neutral fat rises accompany all hypercholesteremic states, studies were done first with both these lipids together(6) and then with each lipid separately(7). The results showed that a prior plasma accumulation of either neutral fat or phospholipid was quickly succeeded by hypercholesteremia.

In the present study, the role of the liver in the hypercholesteremia induced by prior elevation of plasma phospholipid was investigated. This type of hypercholesteremia could be produced in the complete absence of functioning liver tissue.

I. *Cholesterol content of liver before and after induction of phosphatide induced hypercholesteremia.* To determine whether cholesterol accumulating in plasma following sustained hyperphospholipidemia(7) was accompanied by hepatic depletion of cholesterol, the following experiments were done.

Methods. Fifty male rats of Long Evans strain (Wt: appx. 315 g) between 14-18 weeks of age and which had been starved for 18 hours were used. Fifteen of these were injected intravenously with 3.0 ml of a 2% soybean phospholipide† solution containing 5% dextrose, and then injected continuously at an approximate rate of 1.0 ml per hour for 24 hours with the same concentration of phosphatide solution. Plasma samples obtained before and 24 hours after the beginning of

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† This phospholipide mixture was furnished by Glidden Co., labeled "Soya Lecithin, 'oil-free' RG Brand." Prior to injection, the soy phosphatides were freed from $Ca_3(PO_4)_2$. Phosphatides were further purified before injection into 10 of the 15 rats by successive reprecipitation from diethyl ether and from glacial acetic acid. The reprecipitated product showed no apparent sterol content by our analytical methods.

TABLE I. Cholesterol Content of Rat Plasma and Liver after Phosphatide Injection.

No. of rats	Avg wt (g)	Vol infused (ml)	Plasma total cholesterol (mg/100 ml) Before	Plasma total cholesterol (mg/100 ml) 24 hr after	Plasma phospholipid (mg/100 ml) Before	Plasma phospholipid (mg/100 ml) 24 hr after	Liver cholesterol: Dry wt liver (g)	Liver cholesterol: mg/100 g tissue	Liver cholesterol: mg/organ
			Control rats untreated						
15	307						*	859 ± 29†	29*
			Control rats injected with dextrose solution						
20	318	23	59	66 ± 3.9†	113	118 ± 3.1†	2.86	891 ± 17	26 ± 0.8†
			Rats injected with 2% phosphatide solution						
15	316	25	56	158 ± 12	110	408 ± 44	2.68	1200 ± 49	32 ± 0.8

* Analysis of single liver lobe only, except 5 animals where whole liver was used.
† ± S.E.

infusion were extracted with 1:1 alcohol:acetone and the extract analyzed for total cholesterol(8,9), and phospholipid(10). The liver was removed at 24 hours and cholesterol content determined as described previously for feces(11). Twenty control rats were treated in a manner identical to the above rats except that their infusion consisted of only 5% dextrose. The remaining 15 rats served as untreated controls.

Results. Continuous infusion of phosphatide solution into the 15 rats led to elevation of average plasma phospholipid content (see Table I) from 110 to 408 mg per 100 ml in 24 hours. The average plasma cholesterol also rose from 56 to 158 mg per 100 ml (over 180%) during this same period. Plasma phospholipid and cholesterol of control rats receiving dextrose solution (Table I) showed no significant change. The initial average cholesterol concentration of livers (as estimated by the analyses of the livers of the untreated rats) was approximately 859 mg per 100 g of liver, but after 24 hours of sustained hyperphospholipidemia, the average cholesterol concentration of the liver had increased to 1200 mg per 100 g. Infusion with dextrose alone did not change the liver cholesterol concentration significantly (Table I).

II. *Excretion of biliary cholesterol and cholate during phosphatide induced hypercholesteremia.* To determine whether such hepatic excess cholesterol concentration resulted from some intrinsic change in sterol metabolism within the liver, the excretion of bile cholesterol and cholate were measured.

Methods. The bile ducts of 6 rats were cannulated and bile collected while the animals were confined in a Bollman(12) type cage for 12 hours. During this same period 3 of the rats received 2% phosphatide solution continuously by vein. The remaining 3 rats served as controls receiving 5% dextrose solution. Plasma samples obtained at the beginning and at the end of infusion were analyzed for total cholesterol and phospholipid. The bile was analyzed for total cholesterol by extracting(13) or by direct extraction with alcohol:acetone, precipitation of the cholesterol as the digitonide, and analysis of the digitonide(9). It was analyzed for bile acid by absorption spectrophotometry(14).

Results. The induction of hypercholesteremia by elevation of plasma phospholipid did not appear to be accompanied by any significant change in the production of either bile cholesterol or cholate (Table II). The average biliary cholesterol excretion of hypercholesteremic rats was 1.6 mg in 12 hours as compared to 1.8 mg in control rats. Biliary excretion of cholate also appeared to be the same in both groups. This evidence suggests that the normal course and rate of cholesterol metabolism by the liver remains unchanged in agreement with previous findings(15,16).

III. *Effect of subtotal and total (functional) hepatectomy upon phosphatide induced hypercholesteremia.* The preceding data strongly suggested that excess of cholesterol found in plasma was not arising from liver but was itself effecting a rise in liver cholesterol. The following experiments therefore were done to explore this possibility.

Methods. A series of 20 rats were sub-

TABLE II. Effect of Phosphatide Injection on Bile Cholesterol and Cholate Content in Rats; 3 Rats in Each Series.

Wt (g)	mg phos. inj.	Plasma cholesterol Before (mg/100 ml)	Plasma cholesterol 12 hr (mg/100 ml)	Plasma phospholipid Before (mg/100 ml)	Plasma phospholipid 12 hr (mg/100 ml)	Bile vol (ml)	Bile cholesterol mg/100 ml	Bile cholesterol mg/12 hr	Bile cholate mg/100 ml	Bile cholate mg/12 hr
					Rats given phosphatides					
307	282	60	145	107	346	6.7	23	1.6	230	14
					Rats given dextrose					
309	None	63	73	123	167	7.7	27	1.8	192	14

jected to complete, and 8 rats to subtotal, hepatectomy by previously described methods (17). Immediately after hepatectomy, all rats were given intravenously 1.5 ml of 2% phosphatide solution in 5% dextrose and then infused with the same solution at an average rate of 1.0 ml per hour. Infusion was continued in totally hepatectomized rats for 6 hours and in partially hepatectomized rats for 12 hours. Plasma samples obtained before, and at the end of, infusion were analyzed for cholesterol and phospholipid. In some experiments, packed red blood cell volume was determined at both the beginning and the end of infusion. No significant change in hematocrit was observed. For control purposes, 15 normal rats were subjected to the same program.

Results. A rise in plasma cholesterol quickly occurs after infusion of soy phosphatides into the partially or totally hepatectomized animal (Table III). As a matter of fact, average plasma cholesterol content of the totally hepatectomized rat increased more (46 to 127 mg per 100 ml; an increase of approximately 176%) after 6 hours of infusion than that of the infused normal rat (56 to 96 mg per 100 ml; an increase of approximately 71%). Similarly average plasma cholesterol of the partially hepatectomized rats increased more (56 to 197 mg per 100 ml; an increase of approximately 250%) after 12 hours of infusion than that of the normal rat (56 to 131 mg per 100 ml; an increase of approximately 134%) after the same period of infusion.

Discussion. The above findings confirm our earlier studies(7) indicating that an hyperphospholipidemia induced by *continuous* infusion of mixed phosphatides sufficient to maintain a continuously high level of plasma phospholipid quickly leads to hypercholesteremia. Furthermore, the degree of hypercholesteremia is dependent seemingly not upon the amount of phosphatide infused but upon the excess amount persisting in plasma. Thus our hepatectomized animals received no more phosphatide than control animals but because of their relative inability to rid plasma of phospholipid administered, a greater accumulation of these substances occurred in plasma and this in turn presumably provoked a more severe hypercholesteremia. The above studies also indicate that the excess cholesterol present in hypercholesteremia

TABLE III. Plasma Cholesterol Content of Hepatectomized Rats after Phosphatide Injection.

No. of rats	Avg wt (g)	Avg % liver remaining	Total cholesterol (mg/100 ml) Before inj.	Total cholesterol (mg/100 ml) 6 hr after inj.	Total cholesterol (mg/100 ml) 12 hr after inj.	Total phospholipid (mg/100 ml) mg % lipid phosphorus × 25 Before inj.	Total phospholipid (mg/100 ml) mg % lipid phosphorus × 25 6 hr after inj.	Total phospholipid (mg/100 ml) mg % lipid phosphorus × 25 12 hr after inj.
				Control rats with intact livers				
15	306	100	56	96 ± 4.5*	131 ± 15*	103	206 ± 7.4*	219 ± 42*
				Rats with subtotal hepatectomy				
8	313	33	56		197 ± 20	87		420 ± 44
				Rats with complete hepatectomy				
20	305	None	46	127 ± 6.9		107	632 ± 43	

* ± S.E.

induced by injection of soy phosphatides is apparently derived primarily from extrahepatic sources. Furthermore, unlike other forms of hypercholesteremia (*e.g.,* nephrosis (3), biliary obstruction(1) and Triton induced hypercholesteremia(5)), the liver of the rat with phosphatide induced hypercholesteremia demonstrates an increased concentration of cholesterol. These findings are explainable if it is remembered that in phosphatide induced hypercholesteremia plasma phospholipid content is elevated by infusing phosphatide at a rate faster than the ability of liver to remove phospholipid. Removal of some phospholipid by liver takes place as usual(18). During this continual normal hepatic removal of phospholipid, probably some of the excess plasma cholesterol is also removed.

Since, according to the present studies, intravenously administered phosphatide is capable of mobilizing cholesterol from extrahepatic sources and depositing at least some of this cholesterol in the liver of the normal animal, the administration of phosphatides appears to have therapeutic implications. Of course the administration of phosphatide to an animal already exhibiting a deficiency in its phospholipid turnover and a consequent hyperphospholipidemia would only intensify both the latter and the hypercholesteremia it also had evoked(7). Phosphatide then may well be a double edged sort of physiological sword in that it evokes hypercholesteremia when its own egress from plasma is faulty but conversely can mobilize and bring extrahepatic cholesterol to the liver for disposal if its own egress is assured.

Summary. (1) If sufficient phosphatide is intravenously administered to the rat to induce a *continuous* state of hyperphospholipidemia, a state of hypercholesteremia also ensues. The hepatic concentration of cholesterol also is increased. (2) Phosphatide induced hypercholesteremia appears independent of sterol metabolism of liver as judged by assay of bile for cholesterol and cholate contents. Furthermore, total or partial hepatectomy increased the degree of hypercholesteremia induced by experimental hyperphospholipidemia. (3) Mobilization of extrahepatic cholesterol and its subsequent deposition in the liver by experimental induction of hyperphospholipidemia appears to have possible therapeutic implications.

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Estimation of Water Transfer from Amniotic Fluid to Fetus.* (22511)

MARY JANE GRAY, EARL D. NESLEN AND ALBERT A. PLENTL.
Department of Obstetrics and Gynecology, College of Physicians and Surgeons and Presbyterian Hospital, N. Y. City.

The average exchange rate of water between amniotic fluid and maternal system including the fetus amounts to about 27 mols per hour(1). The pathway and mechanism of this exchange is unknown and it is not clear whether the fetus participates to any appreciable degree. If the fetus plays a major role in the transfer of water from amniotic fluid to mother, the treatment of experimental data must provide for this by its inclusion as a separate, third compartment. The theoretic background for such a multi-compartment system in which an exchange takes place between all of them, has been given previously, where a hydrodynamic model was used to demonstrate how transfer rates can be calculated from time activity curves.

The human fetus is inaccessible during pregnancy and samples of fetal body fluids can only be obtained at the termination of the experiment, *i.e.*, at delivery. Since the change in tracer concentration as a function of time follows a double exponential curve for all 3 compartments, a single value for the fetal compartment is not sufficient to calculate transfer rates. A rough estimate of one of these transfer rates can be made from the observation that the time activity curves for the fetus pass through a maximum. The present experiments with hydrodynamic models, analogous to the biologic system, were carried out in an effort to estimate the quantity of water passing from amniotic fluid to fetus per unit time.

Methods. A. *Hydrodynamic models.* The apparatus was essentially that described by Plentl and Gray(2). Amniotic fluid-, maternal-, and fetal compartments were represented by glass tanks containing 1,500, 30,000 and 1,500 ml of water. Six small laboratory pumps were used to transfer the water in the 6 possible directions. "Time activity" curves were obtained by following the change in concentration of a dye added to the amniotic fluid compartment. A series of experiments was then carried out by varying the transfer rates from the amniotic fluid to the other 2 compartments (mother and fetus) but keeping their sum constant. The rates were so adjusted that a total of 500 ml entered and left the amniotic fluid compartment per hour. In 4 representative experiments 25, 50, 75 and 100% of this amount passed from amniotic fluid to fetus. The remaining transfer rates were changed slightly in each experiment so as to keep the system in a steady state. B. *Clinical.* Total body water determinations were carried out on pregnant volunteers at term 2 days before carrying out the experiments. All patients had been admitted for elective Cesarean section. On the day of the operation a trans-abdominal amniotomy was performed by a technic previously described(3). An accurately known amount of deuterium oxide was then introduced into the amniotic sac after a control sample of amniotic fluid and maternal blood was taken. The tracer and amniotic fluid were then thoroughly mixed by frequent withdrawal and reintroduction of the fluid, change in the position of the patient and massage. At the time of section samples of maternal serum, amniotic fluid and cord blood were obtained. These were analyzed for their deuterium oxide content using the falling drop technic.

Results. The exchange rate between fetus and mother is probably in the neighborhood of 2,000 to 3,000 ml per hour and the amount of water entering and leaving the amniotic fluid is known to be about 500 ml per hour (4). The water can be transferred from the amniotic fluid to the mother or the fetus or both. When the contribution to each of these compartments was varied only minor differ-

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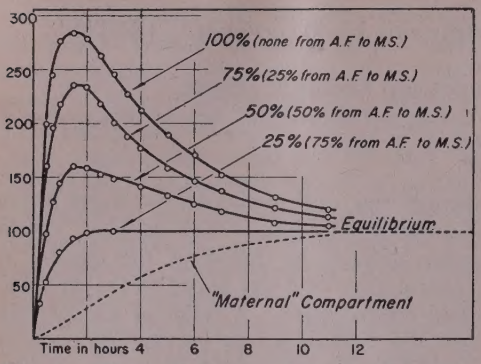

FIG. 1. Time activity curves for the fetal compartment obtained on hydrodynamic models under conditions described in the text. Total amount entering and leaving amniotic fluid compartment remained constant but percentage passing through fetus varied as indicated.

ences were noted in the time activity curves for amniotic fluid and maternal systems but an appreciable variation was observed for the fetal compartment. No matter how the other transfer rates were varied the time activity curves for the fetus never exceeded the equilibrium value if less than 25% of the amount leaving the amniotic fluid went through the fetus. The fetal curves invariably passed through a maximum when more than a quarter of the transfer took this route. The results are recorded graphically in Fig. 1.

During previous investigations on the water transfer in this system the clinical experiments were carried out over as long a period as possible. Hourly samples of amniotic fluid and maternal blood were collected over periods of at least 6, usually 10 hours. The concentrations of deuterium oxide in cord blood and maternal system at the end of this time were always identical, an expected finding in view of the rapid exchange of water across the placenta.

TABLE I. Summary of Results Obtained in 5 Normal Patients. Cesarean section was performed within a period of one hr after amniotomy.

Elapsed time after inj. of D_2O into AF (min.)	AF vol (ml)	Calc. equil. value (% D_2O)	% equilibrium value	
			Maternal serum	Cord blood
20	790	.123	7	236
22	1139	.129	9	123
25	660	.118	17	204
50	690	.158	40	155
60	500	.205	33	350

The present experiments were of much shorter duration. The Cesarean sections were carried out within 15 to 60 minutes after the introduction of deuterium oxide into the amniotic fluid compartment. The results on 5 of these experiments are reproduced in Table I. The deuterium oxide concentration in cord blood invariably exceeded the calculated equilibrium value by 25 to 250%. Since the time concentration curve for the fetus eventually approaches the equilibrium value, this curve must pass through a maximum. In the light of the observations on hydrodynamic models described above, this must be interpreted as evidence that a minimum of 25% and probably more than 50% of the water transfer from amniotic fluid to mother is accomplished through the intermedium of the fetus.

Summary. Deuterium oxide was injected into the amniotic sac of pregnant women one hour or less before delivery by Cesarean section. The tracer concentration in cord blood invariably exceeded the calculated equilibrium value. This observation led to the conclusion that time activity curves for the fetal compartment must pass through a maximum. On the basis of experiments with hydrodynamic models this was interpreted to mean that at least 25%, probably more than 50% of the water transfer from the amniotic fluid to mother is accomplished through the intermedium of the fetus.

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Influence of Estrogens on Total Serum Copper and Caeruloplasmin.* (22512)

ELLA M. RUSS AND JULIE RAYMUNT. (Introduced by David P. Barr.)
Department of Medicine, New York Hospital-Cornell Medical Center, New York City.

Studies in this laboratory involving the separation of low-density lipoproteins from Cohn Fraction I + III of human plasma have led to observations on the copper-bound protein, caeruloplasmin.

Methods. Plasma of human subjects, before and after a course of estrogen therapy, was fractionated according to method No. 10 of Cohn and his associates(1) as adapted in this laboratory(2). Fraction I + III containing all of the low-density lipoproteins, almost all of the caeruloplasmin, fibrinogen and trace amounts of other proteins, was then brought to a solution density of 1.063-1.070 with NaCl and centrifuged at 30,000 rpm in a Spinco Model L centrifuge for 21 hours at 5°C. All lipoproteins were thus floated to the top in a characteristic yellow, fatty layer while the remaining proteins were densely packed in a blue to blue-green layer in the bottom of the tube. The blue color was attributed to caeruloplasmin, and its intensity to the concentration of the copper-bound protein. It was consistently noted that the blue color of this layer in Fraction I + III from equivalent amounts of plasma was always more intense after subjects had received estrogen. Thus we were prompted to make quantitative measurements for copper and caerulopasmin before and after the hormone was given. Realizing the probability that caeruloplasmin was present in Fraction IV + V + VI as well as Fraction I + III of the Cohn method number 10 and that recoveries of minute amounts might not be ideal, analyses for copper were carried out on unfractionated serum. The diethyldithiocarbamate colorimetric method of Gubler and co-workers(3,4) was employed. With their procedure they demonstrated that all copper not complexed as caeruloplasmin reacts directly with the carbamate. This is termed the "direct" reacting fraction and normally accounts for only about 0-10% of the total serum copper. The "indirect" reacting fraction, or that portion not reacting directly with carbamate, presumably corresponds to caeruloplasmin and normally represents the bulk (90-100%) of the total copper. Markowitz, Gubler, Mahoney, Cartwright and Wintrobe (5) showed that concentrations of caeruloplasmin calculated from the copper values of the "indirect" fraction were in good agreement with those they obtained from immunochemical and Warburg manometric technics using paraphenylenediamine as substrate.

Results. Table I shows the results for total, "direct" and "indirect" copper concentrations in unfractionated serum from a variety of patients before and after a course of estrogen. It includes diagnoses and also dose and duration of estrogen treatment. None of the subjects had received any other form of drug therapy, either immediately preceding or during the experiments. None suffered respiratory infections. In all instances there was, after 3 to 4 weeks on 1 mg Estinyl (Ethinyl Estradiol)† o.d., a 1.5 to 2.5-fold increase in both the total and "indirect" reacting copper. Subject *Bla* still showed a 71% increase even after his dose had been lowered to 0.25 mg o.d. for 4 weeks. For purposes of comparison we have shown in the Table copper concentrations of normal males and females(6). Values in our laboratory on 6 normal individuals fell within their normal range.

Hypercupremia has been reported in a variety of diseases for which there is no known common denominator(7). The highest values reported appear to be in acute leukemia and in normal pregnant women(7). Higher values for total and "indirect" reacting serum

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† Kindly supplied by Dr. John Black of Schering Corp., Bloomfield, N. J.

TABLE I. Direct and Indirect Reacting Serum Copper Levels following Estrogen Therapy.

Subjects	Control (copper, γ/100 ml) Total	Direct	Indirect	Estrogen therapy‡ (copper, γ/100 ml) Duration (wk)	Dose (mg, o.d.)	Total	Direct	Indirect
Bla ♂ 39 coronary occlusion	157	25	132	4	1.0	334	52	282
				4	.5	283	77	205
				4	.25	268	47	221
Adi ♂ 49 coronary occlusion	153	15	138	4	1.0	356	34	322
				8	1.0	375	71	304
				15	1.0	522	35	487
Unr ♀ 47 myxedema	131	14	117	3	1.0	340	36	304
Cit ♀ 59 myxedema	254	34	220	4	1.0	374	18	356
Bor ♀ 72 myxedema	150	13	137	2	1.0	202	7	195
Cop ♀ 39 xanthomatosis	152	6	146	4	1.0	325	69	256
				15	1.0	320	93	227
Sta ♀ 49 xanthomatosis	175	24	151	4	1.0	328	29	299
Haa ♀ 56 xanthomatosis	169	0	169	8	1.0	360	39	321
				16	1.0	334	25	309
6 normals (ours)	(101–137)							
*23 normal ♀	116 ± 16							
40 " ♂	105 ± 16							
†10 mixed normals	109 ± 17	5 ± 6	103 ± 11					
10 normal pregnant ♀	257 ± 38	29 ± 13	228 ± 35					

* Reference (6). † Reference (5). ‡ Estrogen given as Estinyl (ethinyl estradiol).

copper were obtained after administration of estinyl than was found in any previously reported condition and even in the third trimester of pregnancy(5). In view of these observations and reports that serum copper concentrations are significantly higher in females than in males(6) the possible interrelationship between the enzymatically active caeruloplasmin and estrogen metabolism is suggested.

Summary. 1. Administration of estrogen as Estinyl (Ethinyl Estradiol) in doses ranging from 0.25-1.0 mg o. d. for 3 to 4 weeks markedly increased the serum total and "indirect" reacting copper or caeruloplasmin levels in 8 subjects with a variety of diseases. 2. Values obtained after 4 weeks on 1 mg Estinyl o. d. were considerably higher than in any previously reported conditions.

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Toxicity of Histamine and Dial with Urethane in Intact and Adrenalectomized Hamsters.* (22513)

LELAND C. WYMAN, LOIS L. DRAPEAU, AND GEORGE P. FULTON.
Department of Biology, Boston University, Boston, Mass.

To select suitable doses of histamine, in a study of the effect of a chemical stress on peripheral circulation in the cheek pouch of the golden hamster (*Mesocricetus auratus*), data on toxicity of this substance in hamsters were accumulated. Since our observations of the circulation during stress with histamine necessitated the use of anesthesia, we have determined the toxicity of combinations of histamine and the anesthetic of choice, dial with urethane, selected because of sustained action from single doses. So far as we know, detailed information on the toxicity of histamine for hamsters has not been reported, and in view of the increasing use of this animal for experimental purposes an account of our work may be useful.

Methods. Tests were performed on 467 male hamsters, 8 to 10 weeks old, weighing 80 to 100 g. Of these, 271 were intact, 166 had been adrenalectomized 4 or 12 days previously, and 30 were sham-operated controls. The histamine used in most of the experiments was the chemically-pure base (Fisher). In previous work this form of histamine gave more uniform results, although more toxic than the acid phosphate (Burroughs-Wellcome) which has been commonly employed in adrenal studies. A few tests with the acid phosphate were done in 34 hamsters. Intraperitoneal injections of 5 to 10% histamine were used in adrenalectomized hamsters and a 20% solution was used for intact hamsters. Anesthesia was induced by dial with urethane (hereinafter referred to as DU), containing 0.1 g diallylbarbituric acid, 0.4 g urethane, 0.4 g monoethylurea per cc (Ciba Pharmaceutical Products, Inc.). Single doses of 0.1 cc per 100 g body weight injected intraperitoneally provided a constant level of anesthesia within one hour and lasting for more than 5 hours. Consequently, the dose of histamine was given one hour after the injection of DU. The body temperature of the animals was maintained during DU anesthesia by contact with glass coils containing circulating warm water. Pretreatment with cortisone consisted of 12 daily subcutaneous injections of a saline suspension of cortisone acetate (Cortone, Merck), 25 mg per cc, diluted when necessary with Aqueous Vehicle No. 1 (Merck). Toxicity tests were performed on the twelfth day. The total mortality in each series at the end of 4 hours after histamine was recorded as a measure of toxicity.

Results. The results of the tests on intact hamsters are summarized in Table I. The LD_{50} at 4 hours for chemically-pure histamine in unanesthetized male hamsters is approximately 75 mg per 100 g body weight. The combination of histamine with DU anesthesia is considerably more toxic (LD_{50} at 4 hours between 20 and 30 mg), although DU alone does not cause death. Pre-treatment with 12 daily doses of 0.3 mg of cortisone appreciably reduced the mortality resulting from 30 mg of histamine per 100 g in hamsters under DU anesthesia, and also provided some protection against 60 mg per 100 g. Pre-treatment with 5 mg of cortisone acetate per day for 12 days provided some protection against the larger doses of histamine in unanesthetized hamsters and against 30 mg per 100 g in the DU group. The smaller doses of cortisone may afford greater protection against histamine and DU intoxication than the larger doses, since 5 mg daily doses are toxic *per se* in the hamster(1).

Adrenalectomy increased the susceptibility of unanesthetized hamsters to histamine poisoning about 10 times (Table II). DU anesthesia alone was toxic to adrenalectomized hamsters, although it did not kill intact hamsters. Very small doses of histamine (0.25

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TABLE I. Toxicity of Histamine and Dial with Urethane in Hamsters with Intact Adrenals.

Procedure	Dose of histamine base (mg/100 g body wt) 0	20	30	40	55	60	65	75	80	90	95	100	Total No. of hamsters
No anesthesia					0* (10)	10 (20)	30 (10)	50 (10)	53 (15)	60 (20)	60 (10)	86 (15)	110
Dial with urethane anesthesia	0 (10)	33.3 (9)	90 (10)	80 (10)	100 (20)	100 (20)							79
Cortisone, 5 mg/day, for 12 days; no anesthesia						0 (10)		11 (9)				33.3 (9)	28
Cortisone, 5 mg/day, for 12 days; DU anesthesia	0 (10)		60 (10)			100 (10)							30
Cortisone, 0.3 mg/day, for 12 days; DU anesthesia	0 (9)		20 (10)			80 (10)							29
Histamine phosphate; no anesthesia												60 (5)	5

* Upper figure, % mortality within 4 hr; lower figure in parenthesis, No. of animals.

TABLE II. Toxicity of Histamine and Dial with Urethane in Adrenalectomized Hamsters.

Procedure	Dose of histamine base (mg/100 g body wt) 0	.25	.5	2	5	8	10	12	15	20	Total No. of hamsters
No anesthesia				0* (7)	18 (16)	66 (18)	86 (15)	60 (10)	100 (6)	100 (9)	81
DU "	78 (14)	100 (4)	100 (9)	100 (9)							36
Cortisone, 1 mg/12 days; DU anesthesia	0 (10)	0 (10)									20
Histamine phosphate; no anesthesia					0 (9)		20 (10)			50 (10)	29
Sham-operated controls; no anesthesia					0 (10)		0 (15)			0 (5)	30

* Upper figure, % mortality within 4 hr; lower figure in parenthesis, No. of animals.

mg per 100 g) combined with DU anesthesia were 100% lethal for adrenalectomized hamsters. Considerably larger doses (5, 10 and 20 mg per 100 g) did not kill any of the sham-operated controls. Histamine acid phosphate was likewise much more toxic to adrenalectomized hamsters. Pre-treatment with 12 daily doses of 1 mg of cortisone acetate provided complete protection within the 4-hour observation period, both against DU anesthesia alone and DU with 0.25 mg of histamine per 100 g.

The magnitude of susceptibility of the golden hamster to intoxication by intraperitoneal histamine is about the same as that of rats(2) or of mice(3). Consequently, the hamster may be considered as one of the more resistant species. Furthermore, the increase in susceptibility following adrenalectomy resembles that found in rats(4,3). The histamine tolerance test should, therefore, be useful in studying adrenal insufficiency in hamsters.

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Comparative Study of Soluble Proteins of Corneas from Three Mammalian Species.* (22514)

Marion A. Guidry, Joyce B. Kelly, and James H. Allen.
(Introduced by William B. Wendel.)
Departments of Ophthalmology and Biochemistry, Tulane University School of Medicine, New Orleans, La.

There have been few studies on the nature of the water soluble proteins of corneal tissue. The first report on the composition of the soluble proteins of cornea(1) showed that there is a small amount of water extractable protein in bovine corneas equal to approximately 0.15% of the wet weight of this tissue. Temperature denaturation studies suggested that these extracts contained 2 distinct globulins in addition to an albumin.

The present study was undertaken to determine whether any similarity exists in the composition of extracts of corneas from various species. Sedimentation studies were conducted to determine the number of protein fractions and the particle size distribution of these fractions in the various extracts.

Materials and methods. A. *Source of Corneas.* The swine, bovine, and New Zealand White rabbit corneas were obtained soon after death of the animals. The corneas were removed, washed in 1% saline solution to remove any adhering blood and then placed on cracked ice until the collection was complete. Within 4-5 hours the corneas were either immediately extracted or frozen until extraction could be accomplished. The corneas from the mongrel rabbits were obtained from rabbit heads which were frozen immediately after death of the animals and shipped in the frozen state. Removal of these corneas occurred before the tissue thawed completely. B. *Extraction.* Extracts were made by adding the tissue to the phosphate buffer and mincing for 15 minutes at 10°C in an Eskimo Blender, producing a gel from which the extract could be separated by centrifugation at 110,000 x g for 5 minutes. The precipitate was then extracted once more by the same procedure and the 2 extracts combined. When mincing swine, bovine, and mongrel rabbit corneas, 100 ml of buffer and 25 g of tissue were used for each extraction. When mincing New Zealand White rabbit corneas, 24 ml of buffer were used for each 2 g of corneal tissue. C. *Concentration of proteins in extracts.* Two methods of concentrating the extracts were employed. One method consisted of complete precipitation of all proteins in the extract by saturating with ammonium sulfate. The precipitated protein was redissolved in a small amount of buffer and dialyzed in the cold against pure buffer until the solution was free of sulfate. The second method of concentrating the extracts consisted of centrifuging in a preparative rotor for 5 hours at a mean gravitational field of 173,400 x g. This sedimented the proteins so that they were contained in the lower half of the solution in each centrifuge tube. The upper half of the solution in each centrifuge tube (which was protein free) was removed and discarded. The remaining extract was combined and the same procedure applied 2 more times, thus increasing the concentration of the proteins of the original extract by a factor of 8. D. *Sedimentation constants.* The sedimentation constants of the proteins in the above concentrates were determined with the Specialized Instruments Co. Model E analytical ultracentrifuge at a speed of 52,640 rpm (201,320 x g). The standard Spinco Analytical Rotor "A" was employed. The temperature of the rotor was 24° to 25° C. Photographs of the sedimentation diagrams were taken at 16 minute intervals after reaching full speed. A microcomparator was utilized to measure the boundaries on the photographic plates. The sedimentation values reported were determined in the phosphate buffer described at 24-25°C and are not

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TABLE I. Sedimentation Constants of 3 Soluble Protein Fractions of New Zealand White Rabbit Corneas Obtained under Various Conditions of Preparation.

Source of protein	Method of concentration of extract	S × 10^{13} I	II	III
Fresh corneas	Ammonium sulfate precipitation	a. 3.2	3.9	5.9
		b. 3.1	4.1	6.2
Fresh "	Ultracentrifugal	3.3	4.1	6.3
Frozen "	Ammonium sulfate precipitation	3.0	4.0	6.2

corrected to standard conditions. E. *Concentration measurements.* The areas under the curve in the sedimentation diagrams, which are proportional to the concentration of the individual protein fractions, were measured by planimetry.

Results. A. *Effect of concentration methods on proteins obtained.* The effect of the 2 methods of concentrating the extracts from New Zealand White rabbit corneas on the amount and nature of the proteins in the final solutions was investigated. It was observed in all preparations that 3 protein fractions were obtained from this tissue. The sedimentation values of the 3 components in the mixtures were measured for extracts concentrated by the 2 methods (Table I). It was observed that freezing the corneas for as long as 3 months before extraction, or concentrating the extracts by either the ammonium sulfate precipitation method or the centrifugal method had no significant effect on the sedimentation values of the 3 protein components in the mixture. These components are labeled I (lightest), II (intermediate) and III (heaviest component). The relative concentrations of the protein fractions in the various preparations, based on the measurement of the areas under the respective curves on the schlieren patterns were determined (Table II). The differences in concentration for the fractions in the various preparations were within experimental error and therefore are not significant. Similar studies with extracts from ox cornea also suggest that tissue may be frozen for a considerable length of time before extraction without affecting the amount or the nature of protein extracted. The method of concentrating these extracts also had no effect on the proteins in the final preparation.

TABLE II. Relative Concentrations of Protein Fractions of Various Preparations of Soluble Proteins from Fresh Corneas of New Zealand White Rabbits.

Method of concentration of extract	Fraction	% of total area of Schlieren diagram
Ammonium sulfate precipitation	I	46–51
	II	24–21
	III	24–26
Ultracentrifugal	I	53
	II	18
	III	27
Ammonium sulfate precipitation	I	54
	II	21
	III	26

B. *Comparison of particle size distribution of soluble corneal proteins from 3 species.* The sedimentation constants (each an average of 4 separate determinations) for the protein components in bovine, swine, New Zealand White and mongrel rabbit corneal extracts are shown in Table III. The preparations from mongrel rabbit corneas showed a difference in the number of protein fractions as compared to preparations from New Zealand White rabbit corneas. Only 2 definite fractions could be distinguished. The lighter fractions from both rabbit preparations showed similar sedimentation characteristics. The second fraction from the mongrel rabbit preparations, however, sedimentated at a rate significantly greater than either the second or third fractions of the New Zealand White rabbit preparations.

The bovine preparations also contained 3 components. Two of these showed sedimentation constants comparable to the second and third components of the New Zealand White rabbit preparations; the third component, however, was considerably heavier and showed the highest sedimentation constant of all components in the preparations

TABLE III. Distribution of Protein Components in Extracts of Corneas from Various Sources.

Source of tissue	S × 10^{13}
NZW rabbits	3.2, 4.0, 5.9
Bovine	3.7, 6.3, 16.6
Swine	2.2, 3.3, 5.8, 11.7
Mongrel rabbits	3.0, 7.7

studied. The swine cornea preparations contained a total of 4 components, 2 of which sedimented at rates different from components in the other preparations. The above sedimentation values for the components in the mixtures cannot be taken as sedimentation coefficients of the respective components because these values were not corrected to conditions which would obtain in a theoretical medium having the density and viscosity of water at 20°C at zero protein concentration (standard conditions).

Conclusions. These studies show that there are fundamental differences in number of protein fractions and particle size distribution in extracts of water soluble corneal proteins from 3 different mammalian species. A marked difference was also noted in number of fractions and particle size distribution in preparations from New Zealand White rabbits and mongrel rabbits.

Summary. 1. Methods of concentrating soluble proteins are reported for aqueous extracts of cornea. These methods apparently do not denature the proteins. 2. These methods were applied to increase concentration of protein from aqueous extracts of bovine, swine, New Zealand White rabbit and mongrel rabbit corneas to a level which would permit ultracentrifugal analysis of these extracts. 3. Fundamental differences in number of protein fractions and particle size distribution were noted in these extracts. This suggests a species difference in soluble corneal proteins. 4. Differences were noted in sedimentation diagrams of extracts from New Zealand White rabbits and mongrel rabbits, suggesting differences in soluble corneal protein content of different strains of the same species.

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Comparative Fat and Fatty Acid Intestinal Absorption Test Utilizing Radioiodine Labeling—Results in Normal Subjects.* (22515)

JAMES R. MALM, KEITH REEMTSMA,† AND HAROLD G. BARKER.
Department of Surgery, Columbia University, College of Physicians and Surgeons, and Surgical Service, Presbyterian Hospital, New York City.

Disorders of intestinal absorption may be divided arbitrarily into two groups: (a) those with deficiency of digestive enzymes; and (b) a group with normal enzyme pattern but impaired absorption due to intestinal dysfunction. The most direct way to distinguish between (a) and (b) is by analysis of duodenal contents for enzymes(1,2). Another method, admittedly indirect, involves measurement of products of digestion and absorption in the blood. In theory, a person with a normal enzyme pattern and intestinal absorption would show a comparable response to ingestion of both unhydrolyzed and hydrolyzed substances; a person lacking enzymes but possessing normal absorptive ability should show a normal response only to the hydrolyzed form. This hypothesis has been thoroughly tested in starch-sugar(3) and in protein-amino acid(4,5) tolerance tests. We have devised a similar test for fats utilizing I^{131}-labeled fats and fatty acids. This report describes the technique and results of its use in normal human subjects.

Methods. The whole fat selected was commercial olive oil since it is not unpalatable, has a high iodine number, 79-88, and an absorption coefficient of 98. It consists of approxi-

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† Postdoctoral Research Fellow, National Cancer Institute, U. S. Public Health Service.

mately 75% glycerine trioleate and its major fatty acid, oleic acid, can easily be obtained and iodinated. By a modification of previous methods(6), we have carried out the iodination as follows: one ml of 1% sodium iodide containing 500 μc of I^{131} is added to approximately 50 ml of acidified chloroform. This purple-colored solution is extracted with additional chloroform and the iodine is then halogenated by a stream of chlorine gas forming iodine monochloride (Wijs' method). The end point of halogenation is the disappearance of the purple color. Eighty grams of olive oil or oleic acid in an equal volume of chloroform are then added and the solution is allowed to stand for 24 hrs after which it is washed 5 times with 60 ml amounts of 10% sodium carbonate in water removing any free iodine and precipitating any free fatty acid. Oleic acid is washed with 10% sodium thiosulfate instead of carbonate. The solution is dried over anhydrous sodium sulfate and filtered until clear. The chloroform is then evaporated under vacuum while bubbling nitrogen through the solution. The resultant iodinated oil or fatty acid is clear and is odorless with respect to chloroform. Eighty % of the initial radioiodine can be expected to become bound to fat or fatty acid with the remainder lost in the washings. Twenty-seven patients without evidence of gastrointestinal disease served as subjects. Lugol's Solution (10 drops b.i.d.) was administered for 2 days prior to the test to saturate the thyroid. $Iodine^{131}$ tagged olive oil was used as the test material in 7 subjects and oleic acid in 20. Following an overnight fast the test material (5-10 ml containing approximately 50 μc of radioactivity) was administered by mouth, the precise volume ingested being determined by difference in weight of the vessel. One hour later breakfast was given and the subjects were allowed to be ambulatory. Blood, urine, and stool were collected at frequent intervals up to 72 hours (Table I). Serums were separated and stools prepared by homogenization. Samples were pipetted into counting tubes and counted to an accuracy ($\pm$ 1.S.D.) of 2% or better in a well type scintillation counter, proper decay factors and coincidence corrections being applied when necessary. Dilution of the standards for counting was carried out in ether. All results were expressed in percentage of total dose given. Figures for serum samples represent percentage of the total dose which is in the entire blood stream at the time of sampling and are based on an assumed serum volume of 4.5% of body weight. Urine and stool data are each expressed in cumulative figures calculated into percent of dose administered.

TABLE I. Percentage of Ingested Material in Blood Stream, Urine, and Stool at Various Time Intervals.

	Oleic acid			Olive oil		
	Subjects	Mean	S.D.	Subjects	Mean	S.D.
Serum						
1 hr	15	1.8	.7	6	3.8	1.6
2	18	3.5	1.7	6	9.0	4.0
3	16	5.2	1.9	7	13.1	3.6
4	20	6.7	1.9	7	14.7	4.0
6	20	8.0	2.2	7	11.2	3.6
8	7	7.1	2.3	—	—	—
24	20	2.9	1.1	7	3.3	.7
48	4	1.0	.4	—	—	—
Urine*						
12 hr	13	30.3	10.0	5	47.9	12.6
24	13	54.2	8.9	5	70.3	21.6
48	9	72.3	10.0	—	—	—
72	7	79.2	9.1	—	—	—
Stool						
72 hr	7	14.5	6.2	—	—	—

* Urine figures are cumulative.

Results. The data for our normal subjects are presented in the accompanying table and figure. Of particular interest is the fact that radioactivity appears in the serum at a higher concentration following the ingestion of tagged fat than fatty acid. This is true of all sampling times individually including the peak concentration which reaches 14.7 $\pm$ 4.0 (S.D.) % of dose in the case of fat but only 8.0 $\pm$ 2.2 (S.D.) % for fatty acid. Peak serum concentration is reached in 3½ to 4 hours with fat but not until 5-6 hours with fatty acid. In both cases only 2 to 4% of the dose remains in the blood stream at 24 hours. Urinary excretion is likewise more rapid in the case of fat with some 70% of the radioactivity recovered in the urine within 24 hours but only 55% with fatty acid. More prolonged collection leads to nearly complete

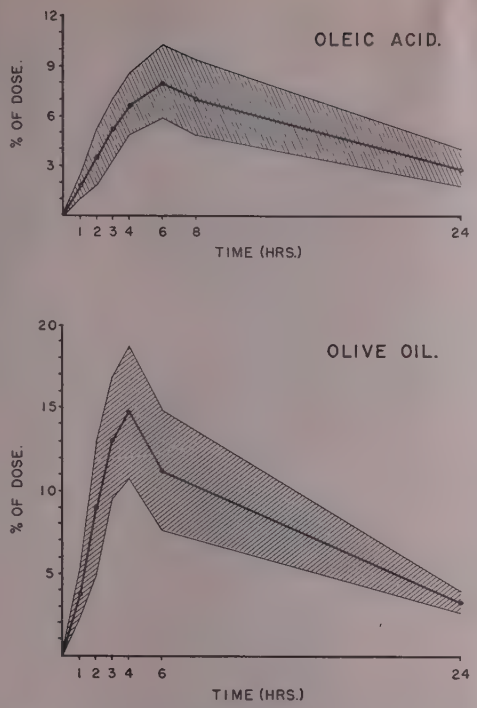

FIG. 1. Percentage of ingested I^{131} dose in circulation at various time intervals. Based on serum volume of 4.5% of body wt. Zero time is time of ingestion. Shaded areas represent plus or minus one standard deviation.

recovery when urine and stool figures are added. Our data for the fat studies agree closely with those of others(6,7). Fatty acid figures have not been previously available in humans to our knowledge.

Discussion. The validity of radioactive-tagged fat technics in the study of intestinal absorption rests on the assumption that the I^{131}-fat complex remains intact during passage through the intestinal membrane. This is probably true since the iodine is incorporated chemically and much higher levels are achieved when carrier-free iodine is given(8). Furthermore, the major part of the iodine is in the lipid fraction of blood in the early stages of absorption(6). If the label remains intact during passage through the intestinal wall then the presence of radioactivity in the blood stream indicates absorption regardless of the durability of the tag after absorption. Unless there is inconsistent extravascular sequestering, it is probable that the serum levels achieved during the early hours of sampling are closely proportional to the actual amount of fat or fatty acid absorbed since disposal is slow at first. It is our current opinion from preliminary analysis of serum curves in about 200 studies in patients with a variety of disorders(8) that serum curves are more informative than cumulative stool or urinary excretion figures. Even without serum figures, however, considerable information can be gained from simple 24 hour urinary excretion figures when the principle of comparative fat and fatty acid absorption is employed. The explanation for differences in serum radioactivity following ingestion of fatty acid and fat in normal subjects is not apparent. Some studies(9-11) have suggested that fatty acids are absorbed by way of the portal system thus presenting them to the liver in high concentration while fats are absorbed by way of the lymphatics. Others (12-15), however, have failed to find any difference in routes of absorption. Possibly gastric emptying time or transit time to level of predominant absorption is different for the two substances. The presence of iodine in the molecule may alter transport across intestinal cells with the more profound effect in the case of the smaller molecule. We do not feel that the 25% portion of olive oil which is made up of molecules other than trioleate is responsible for the differences observed since our results with olive oil are almost identical with those reported for trioleate(7). It is quite possible that the oleic acid is absorbed equally or more rapidly than the olive oil but that differences exist in rate of disposal from the blood stream. Since urinary loss is actually slower following oleic acid, the difference would have to be in more widespread distribution within the body or selective concentration in certain tissues. There is no appreciable uptake by the thyroid in properly prepared patients(8). That the difference may be one of absorption is suggested by the higher fecal recovery following fatty acid administration.

It should be emphasized that the differences between the oleic acid and fat tests in normal

subjects do not lead to confusion when the test is applied clinically. In patients with enzyme deficiencies oleic acid gives higher serum concentrations of radioactivity than does fat(8) which is the reverse of the case with normals. Furthermore, the serum levels observed in abnormals are usually so much lower than those in normals that interpretation is not difficult.

Summary. A test has been proposed for evaluation of intestinal malabsorption states. It involves administration of isotopically tagged whole fat on one day and fatty acid on a separate occasion. By comparison of resultant radioactivity in blood and urine one can judge whether an abnormality found is due primarily to lack of proper digestive enzymes or to intrinsic difficulty with the absorption process. Normal figures with standard deviations are given for serum levels of radioactivity and for urinary and fecal loss. It was found that in normal subjects serum concentrations of radioactivity were lower following ingestion of tagged fatty acid than whole fat and the possible significance of this difference is discussed.

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Effects of Orally Inoculated Bacteria on Gastrointestinal Flora of Newborn Mice. (22516)

CHARLES T. RILEY,* VICTOR J. CABELLI, AND ROBERT KELLER
Department of Microbiology, School of Medicine, University of Missouri, Columbia.

This study was undertaken to ascertain if overwhelming feedings of both normal and abnormal microorganisms at birth would produce any obvious changes in the ecology of the intestinal bacterial flora and in the health and development of white mice. Since part of the normal flora is already established by the fifth day after birth(1), the organisms were fed to newborn mice, in order to eliminate the possibility that the preexisting flora would prevent the establishment of these organisms. Because of the recent interest in *Pseudomonas aeruginosa* and *Proteus vulgaris* as agents of infantile diarrhea, they were chosen as 2 of the test organisms.

Most of the attempts to produce experimental shigellosis have been with young or adult laboratory animals. Since the antagonistic action of the gastrointestinal flora of

* Medical Student Research Fellow, National Foundation for Infantile Paralysis.

these animals has been postulated as one explanation to account for the negative results obtained(2,3), Shigella species were also included in this study.

Methods. Pregnant Swiss albino mice were isolated in separate cages and observed. During the experiments, their diet consisted of Purina mouse pellets. Four to 6 hours after birth, all the newborn mice of a given litter were fed 0.05 ml of a 24 hour nutrient broth culture (app. 5 x 10^7 cells) of one of the following organisms: *Shigella dysenteriae, Shigella paradysenteriae, Proteus vulgaris* and *Pseudomonas aeruginosa* freshly isolated from human infectious processes. *Escherichia coli* and a lactobacillus strain previously isolated from normal adult mice were also used. The organisms were deposited into the oral cavity of each newborn mouse using a tuberculin syringe and an 18 gauge needle from which the bevel had been ground away. The mice were then returned to the cage containing their mother. During the course of the experiments, 13 days, the litters were suckled by their mothers. Each day the mice were weighed and their general health observed. At 2 day intervals through the thirteenth day, one mouse from each litter was sacrificed. Its gastrointestinal tract from the level of the diaphragm to the rectum was aseptically removed, weighed, added to 2.0 ml of sterile saline and homogenized using a Virtis homogenizer. Tenfold serial dilutions of this material were prepared and 0.1 ml of each dilution plated in duplicate on the appropriate media as noted below. Colony counts were done after 48 hours incubation at 37°C. The spread-plate method was used throughout to permit the subculture and identification of all the colony types which appeared. V-8 juice agar, azide blood agar base and MacConkey agar were used routinely to determine the numbers of lactobacilli, Gram-positive cocci and Gram-negative enteric organisms present. In those experiments where the mice were fed with *P. vulgaris,* the agar content of the MacConkey medium was increased to 4% in order to inhibit the spreading of these organisms. Thus, more accurate colony counts were obtained. *Control experiments* on normal mice were conducted as described above except that the newborn mice were fed sterile nutrient broth instead of a bacterial culture.

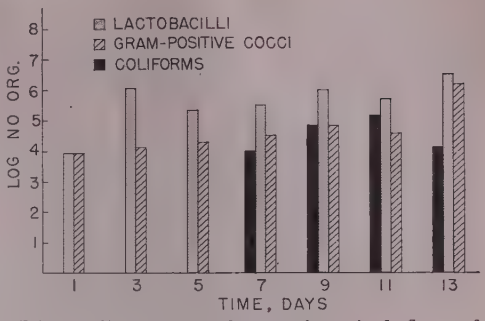

FIG. 1. Emergence of gastrointestinal flora of normal mice.

Results. The sequence of appearance of the normal gastrointestinal flora in the newborn mice was as follows (Fig. 1): Gram-positive cocci appeared within 3 hours after birth. Lactobacilli were observed after approximately 8 hours. Coliform organisms made their appearance about the 7th day after birth. Once established, all the above organisms persisted in quantity during the 13 days of the control experiments. Also, it was found that all the above organisms were part of the normal gastrointestinal flora of adult mice. Lactobacilli and *E. coli* when introduced at birth had no effect on the emergence and persistence of the normal flora and on the growth of the mice. Although superinfection with lactobacilli resulted in initially higher counts of this organism than those with the control animals, by the third day the number of lactobacilli in the superinfected and control animals is the same. Although coliform organisms did not usually appear until the seventh day in the normal animals, premature infections with *E. coli* at birth did establish the organisms at that time. The organisms persisted but did not exceed at any time the number found in the uninoculated mice.

When freshly isolated strains of both S. *dysenteriae* and *S. paradysenteriae* were fed to newborn mice, viable organisms were shown to be present in the G. I. tract 24 hours after oral inoculation. The quantity of organisms recovered at that time indicates that some of

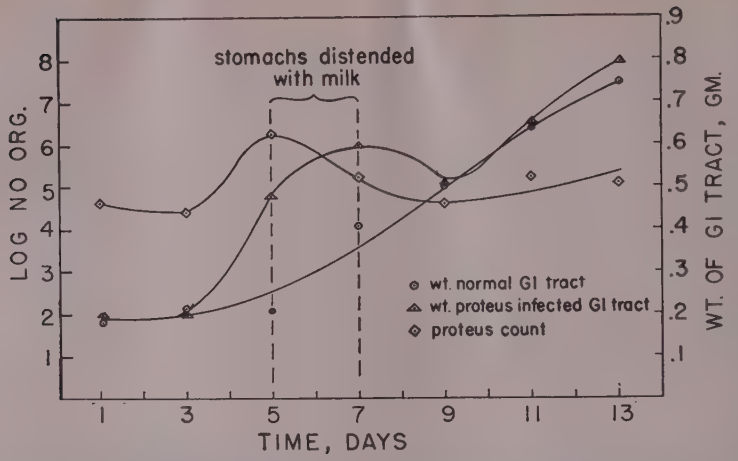

FIG. 2. Weights of gastrointestinal tracts of mice infected with *Proteus vulgaris* in relation to No. of these organisms recovered. Distention of the stomachs in infected animals occurred as indicated.

the organisms were able to pass the gastric barrier and survive in the intestinal tract. However, the number of organisms recovered (2.5×10^3) and the fact that none could be recovered by the third day, suggests a lack of multiplication. The weights, general health and normal flora of these animals did not significantly vary from that of the controls.

Hammond *et al.*(4) found that *Ps. aeruginosa*, when orally inoculated into adult mice, disappeared by the third day in more than 50% of the animals. However, in the experiments described herein using newborn mice, this organism was found to persist in quantity. Thus, the age of the animal must be an important factor in the establishment of this organism in the intestinal tract of mice.

The most marked effect on both the animals and their normal intestinal flora was observed in those groups of mice which were orally infected with *P. vulgaris*. The feeding of overwhelming doses of this organism at birth delayed the emergence of lactobacilli for 4 days. Furthermore, a delay of 6 days in the onset of coliform organisms was also noted in these animals. A striking difference was noted in the weights of the entire G. I. tracts and general appearance of the stomachs. The stomachs of these mice were markedly distended and filled beyond capacity with coagulated milk on about the 5th day and for 2 days thereafter (Fig. 2). These findings coincide with the time of the maximum counts of *P. vulgaris*. On the basis of limited data, it would appear that the gains in weight of these mice occurred at greater rates than those of the control animals (Fig. 3). The weights given represent the total weights less those of the G. I. tracts.

Once established, *Ps. aeruginosa* like *P.*

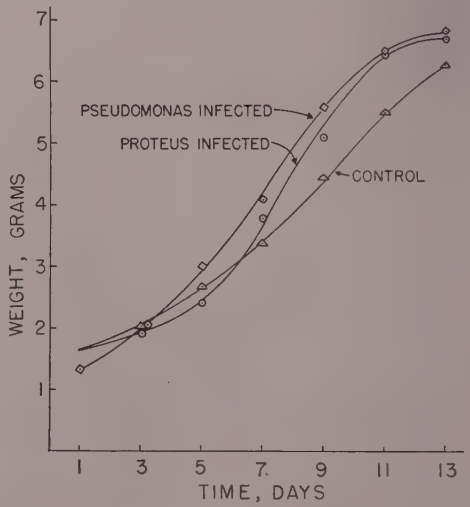

FIG. 3. Gains in wt of mice orally inoculated at birth with *Pseudomonas aeruginosa* and *Proteus vulgaris*. Figures given represent total weights of the animals less those of the G.I. tracts.

vulgaris persisted in the mice during the experiments. Changes observed in these animals were similar to but not as pronounced as those found in the experiments with *P. vulgaris*. Gains in weight greater than those of the control animals were also noted in these mice (Fig. 3).

Discussion. Since the studies on the normal intestinal flora were terminated at time of weaning (13 days), a variable due to change of feeding habits was eliminated. Lactobacilli normally appeared in quantity in 24 hours. Therefore, it was not surprising that superinfection with this organism at birth had no effect. As coliform organisms did not emerge as part of the normal flora till about the seventh day in the control mice, it is of interest that the oral feeding of *E. coli* at birth established this organism at that time, but did not otherwise affect the bacterial ecology. The numbers of each of these 2 organisms recovered from the homologously infected mice did not exceed those found in the control animals. Thus, it would appear that there is an ecological constant for each of these organisms. Inasmuch as *P. vulgaris* and to a lesser extent *Ps. aeruginosa* did inhibit the emergence of both lactobacilli and coliform organisms, it is noteworthy that no permanent adverse effects in these mice were observed. In fact, these animals gained weight at a higher rate than the controls. This observation warrants further investigation. The distension of the stomachs with coagulated milk, which occurred at the time when the viable counts of *P. vulgaris* in the intestines are highest, invites speculation.

Attempts to produce typical shigellosis by feeding the organisms to adult or young animals have been generally unsuccessful(5). Cooper and Keller(6) were unable to infect white mice by gavage with living *Shigella sonnei*. However, repeated feedings of this organism resulted in the development of immunity. In our experiments with *Shigella dysenteriae* and *Shigella paradysenteriae* the organisms gained entrance to the G. I. tract but did not persist even though coliform organisms did not make their appearance until 5 days later. Consequently, in this instance, the resistance of the animals to this infection must be explained by a mechanism other than antibiotic production by *E. coli* as suggested by some workers(2,7).

Summary. The emergence and persistence of Gram-positive cocci, lactobacilli and coliform organisms in the gastrointestinal tracts of Swiss mice from birth to weaning were investigated. Changes in normal bacterial ecology due to oral feedings with both normal and abnormal organisms were described. *Proteus vulgaris* and to a lesser extent *Pseudomonas aeruginosa* delayed the emergence and decreased the quantity of both lactobacilli and *Escherichia coli*. These mice had moderate increases in growth rates over those of the control groups. Recently isolated Shigella species when fed to newborn mice survived passage through the stomach. They did not persist in the G. I. tract nor cause changes in the mice.

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Respiration and Glycolysis of Earle's Strain L Cells.* (22517)

H. J. Phillips and R. J. Feldhaus. (Introduced by C. M. Wilhelmj.)
Department of Physiology and Pharmacology, Creighton University Medical School, Omaha.

In 1943 Earle's Strain L cells(6) after 2 years *in vitro* gave rise to 68% tumors when injected intramuscularly into C3H mice. The incidence of tumors produced in 1946, when cultures were injected, dropped to 1%. Recently it was reported that Strain L cells after 10 years *in vitro* (1950) produced 15% tumors in normal C3H mice and 64% tumors in mice that had been irradiated(13). A metabolic study made in 1942 on the *in vivo* tumors showed that their metabolism was similar to that which had been reported to be characteristic of malignant tissues(2). The purpose of the present work was to determine if the metabolism of Strain L cells today (1954-55) meets the metabolic criteria described by Burk for malignant tissues(2,3,17). No attempt will be made to compare metabolism of *in vitro* Strain L cells to that reported for *in vivo* Strain L tumors(3).

Materials and methods. Initial experiments indicated that about 2 million cells per 7 ml Warburg reaction vessel were necessary to get an accurate record of respiration. For this reason large cultures had to be maintained. Cells were grown on the glass floor of T-60 flasks(4) in culture medium composed of 40% horse serum, 40% Earle's solution and 20% chick embryo extract (50:50). Culture media were changed 3 times weekly. To prepare cells for a series of runs they were carefully washed with Krebs-Ringer-Phosphate solution(16) while still attached to the floor of T-60 flasks. The wash was discarded. A stainless steel nylon brush† was used to dislodge the cells from the flask and cell clusters were dispersed in Krebs solution. The number of cells in a sample was obtained by counting on a hemacytometer(15). Cell number was converted to dry weight in order to conform to terminology of most workers. Dry weight was determined for suspensions of the cells in Earle's solution in the following manner: the number of cells was determined, they were collected by slow centrifugation (500-1000 rpm), dried at 110°C and weighed. The mean weight of one million cells for 3 determinations was 0.46 mg. This value agrees with a calculated dry weight of 0.49 mg if one assumes a dry weight of 11.7%(8) and a mean cell diameter of 20 μ(12). The direct method of Warburg was employed to determine oxygen consumption and carbon dioxide production(16). For the latter determination, potassium hydroxide was omitted from the center well of the reaction vessel, and correction for carbon dioxide retention by the suspending solution was made. Lactic acid was determined by the chemical procedure of Barker and Summerson(1). Samples were run in pairs in an atmosphere of either oxygen or nitrogen. One sample was analyzed immediately after equilibrating for temperature on the Warburg respirometer; the other sample was analyzed one hour later. The difference between samples was taken as lactic acid formation or glycolysis. P-phenylenediamine measures the responsiveness of the cytochrome system in increasing its effectiveness above the natural endogenous rate of oxidation(11). This effect (P-PD), expressed as percent increase in Qo_2, was obtained by adding p-phenylenediamine from the side arm of the reaction vessel so that the final concentration in the reaction vessel was 0.02 M.

Results. In previous experiments on 2-day-old cultures of Strain L cells(10) it had been noted that respiration was fairly high in neutralized horse serum(18), but at the beginning of the present study little or no oxygen uptake occurred in Krebs solution which contained no glucose (Fig. 1). It was desired to make determinations in a physiological saline solution, so that our work would be done under conditions similar to the earlier work on Strain L tumors(3). An isotonic solution of

* Supported by research grant from National Cancer Institute, N.I.H., Public Health Service.

† Brush #61, nylon bristles with stainless steel handle, J. I. Holcomb Mfg. Co., Indianapolis.

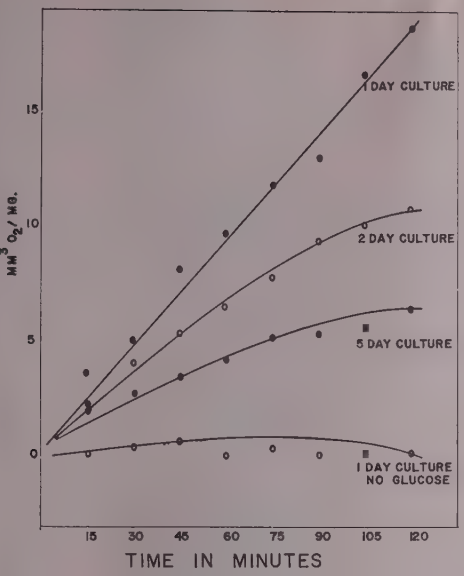

FIG. 1. Effect of media change and glucose on respiration of Strain L cells.

glucose was added to Krebs solution to bring the glucose concentration to 0.1%. Determinations were made in this solution, and respiration was found to be markedly increased by glucose (Fig. 1).

Repeat observations were not the same, and it was then discovered that degree of respiration was related to culture conditions. For example, respiration was found to be highest ($Q_{O_2} = 9.3$) one day following renewal of culture medium (Fig. 1). Respiration was considerably lower on the second ($Q_{O_2} = 6.6$) and fifth day ($Q_{O_2} = 4.3$) following change of culture medium. It was desired to use active viable cells and so only cultures which had been fed the previous day were used for subsequent determinations.

Results of 6 series of determinations are indicated in Table I. A series represents pooled one-day cultures on which all determinations were made at the same time. It was found that oxygen uptake was moderate ($Q_{O_2} = 9.4$) and the respiratory quotient unity. When p-phenylenediamine was added to a suspension of Strain L cells, oxygen uptake increased 251% ($Q_{O_2} = 33.7$). Both aerobic and anaerobic glycolysis was found to be low ($Q_{LA}^{O_2+CO_2} = 1.5$) ($Q_{LA}^{N_2} = 3.5$). Pasteur Effect was calculated by subtracting aerobic from anaerobic glycolysis and was found to be 2.0. Lactic acid formation was not greater than respiration and consequently fermentation excess ($Q_{LA}^{N_2} - 2Q_{O_2}$) was negative (–15.3).

Determinations of aerobic lactic acid formation were made under two conditions: one in which potassium hydroxide was placed in the center well of the reaction vessel and carbon dioxide absorbed, and another in which alkali was omitted and carbon dioxide allowed to accumulate. When carbon dioxide was absorbed, lactic acid formed aerobically ($Q_{LA}^{O_2} = 3.0$), was approximately equal to anaerobic formation ($Q_{LA}^{N_2} = 3.5$). In the presence of carbon dioxide produced by the cells, aerobic lactic acid formation was lower ($Q_{LA}^{O_2+CO_2} = 1.5$). As checked by the test for observations involving non-paired groups(7) there was a significant lowering of aerobic glycolysis due to carbon dioxide ($P < 0.01$).

Discussion. The metabolic pattern which Burk(2) described as characteristic of most malignant tissues emphasized a moderate respiration, a respiratory quotient below unity, a very high anaerobic glycolysis and a high

TABLE I. Respiration and Glycolysis of Earle's Strain L Cells.

Series	Q_{O_2}	R.Q.	P-PD	$Q_{LA}^{N_2}$	$Q_{LA}^{O_2+CO_2}$	$Q_{LA}^{O_2}$
1	8.8 (4)	1.2 (4)		.7 (2)	1.2 (4)	.9 (4)
2	8.6 (2)	.9 (2)		3.4 (2)	.1 (3)	3.7 (4)
3	9.7 (3)	.9 (3)			3.3 (3)	5.1 (3)
4				5.2 (6)		
5	9.7 (3)					
6	9.6 (3)		251 (3)	1.9 (3)	1.5 (2)	2.9 (4)
Mean	9.4	1.0	251	3.5	1.5	3.0

All Q values are expressed as mm³/mg dry wt/hr.
Numbers in parenthesis indicate No. of determinations in each series.
P-PD is % increase in Q_{O_2} on addition of p-phenylenediamine.

aerobic glycolysis. Emphasis was placed also on the slight stimulation of respiration in tumor slices by addition of p-phenylenediamine. Respiration of normal tissues increases greatly (about 200%) when p-phenylenediamine is added.

Our findings indicate that the metabolism of Strain L cells at the present time does not fit Burk's pattern for malignancy. On the contrary, the over-all metabolism of Strain L cells now is more like that of normal tissues(9).

From studies on transplantation(5,6,13) it would appear that malignancy of Strain L cells has changed. They produced 15% tumors in 1950 in non-irradiated mice which was less than it was in 1942(13). Burk reported(17) that the metabolism of 2 lines of cancer cells grown *in vitro* by Sanford(14) paralleled differences in malignancy observed *in vivo*. In view of this evidence, the malignancy of Strain L cells transplanted to C3H mice should be very low if their metabolism *in vitro* parallels their malignancy *in vivo*. If this relation does not occur, then, Strain L cells may be an exception to the statement by Warburg that the respiration of cancer cells never returns to normal(17).

Under aerobic conditions, Strain L cells formed significantly less lactic acid when carbon dioxide was present. Probably the best explanation for this is that pyruvate in the presence of carbon dioxide can be converted to oxaloacetate.

Summary. Earle's Strain L cells were cultured in quantity, so that respiration and glycolysis could be adequately determined. Oxygen uptake was highest the day following renewal of culture medium. Little respiration occurred in the absence of glucose, respiration was moderate ($Q_{O_2} = 9.4$), respiratory quotient was unity (R.Q. = 1.0), anaerobic glycolysis was low ($Q_{LA}^{N_2} = 3.5$), aerobic glycolysis was low ($Q_{LA}^{O_2+CO_2} = 1.5$) and respiration was stimulated 251% when p-phenylenediamine was added. The metabolism of Strain L cells is like that of most normal tissue.

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Effect of Arginine-Vasopressin and Lysine-Vasopressin on Plasma 17-Hydroxycorticosteroid Levels in Man. (22518)

ROGER K. MCDONALD AND VIRGINIA K. WEISE.
National Institute of Mental Health, Bethesda, Md.*

It was previously reported(1) that intravenous infusion of commercial vasopressin (Pitressin) in man causes an increase in the concentration of free 17-hydroxycorticosteroids (17-OHCS) in plasma. Because of the presence of certain unidentified substances in Pitressin reported to cause ACTH release (2,3), it was deemed necessary to study the effect of highly purified vasopressin on plasma 17-OHCS. Two observations are pertinent to this consideration: Guillemin and Hearn showed that Pitressin added to rat anterior pituitary glands in organ culture caused a release of ACTH, whereas highly purified arginine-vasopressin did not(4). On the other hand, Sayers demonstrated that highly purified arginine-vasopressin has the same ACTH-releasing activity as Pitressin in the adrenalectomized rat(5).

Because preliminary observations indicated that injection of arginine-vasopressin caused a smaller increase in plasma 17-OHCS than did Pitressin, the method of preparation of the latter was investigated. It was learned that Pitressin contains arginine-vasopressin and lysine-vasopressin, since both bovine and porcine posterior pituitary lobes are utilized. This observation suggested the possibility that lysine-vasopressin might cause a greater increase in plasma 17-OHCS than arginine-vasopressin. Accordingly, we conducted comparative studies on these 2 compounds.†

Materials and methods. Twelve normal male subjects, ranging from 18 to 31 years of age, were studied. *Test solutions.* (1) 0.9% saline; (2) vasopressin diluent [0.5% chlorobutanol solution adjusted to pH 3.6 with acetic acid]; (3) vasopressin in the above diluent; and (4) Pitressin [Parke, Davis & Co., Lot 909E] in the above diluent. All tests were begun at 8 a. m. with the subjects remaining in bed in a post-absorptive state. Intravenous infusions were given at the rate of 3 ml per minute and consisted of approximately 180 ml of saline plus 4.2 ml of vasopressin plus diluent or vasopressin diluent alone. Injections were given over a period of 1 minute in a volume of approximately 2 ml. *Blood samples.* Venous blood samples of 20 ml were drawn through an indwelling Cournand needle immediately prior to administration of test solution and at intervals thereafter. The heparinized blood was centrifuged and plasma removed and frozen until time of determination. Concentration of free 17-OHCS in the plasma was determined by the method of Silber and Porter(6), as modified by Peterson, *et al.*(7). Duplicate determinations were obtained on all plasma samples. *Experimental design.* The first part of the study was an evaluation of the effect of intravenous infusion of arginine-vasopressin on plasma 17-OHCS. Each of 5 subjects received a one-hour infusion of saline plus vasopressin diluent either preceded or followed by a one-hour infusion of saline plus arginine-vasopressin. On another day the order in which test solutions were administered was reversed. Blood samples were drawn immediately before, and at 30, 60, 90, and 120 minutes after the infusion was begun. The second part of the study was an evaluation of the effect of intravenous injections of Pitressin, arginine-vasopressin, and lysine-vasopressin on plasma 17-OHCS. Four subjects were given 2 units of Pitressin on 1 day and a comparable volume of vasopressin diluent on another day. Eight subjects were given 2 units of arginine-vasopressin on 1 day and 2 units of lysine-vasopressin on another day. The order of administration of test solutions was

* N. I. H., Public Health Service, U. S. Dept. of Health, Education, and Welfare.

† We wish to thank Dr. Vincent du Vigneaud, Cornell University Medical College, for supplying us with highly purified arginine-vasopressin (A-V-N-3 and A-V-N-5) and highly purified lysine-vasopressin (L-V-N-2).

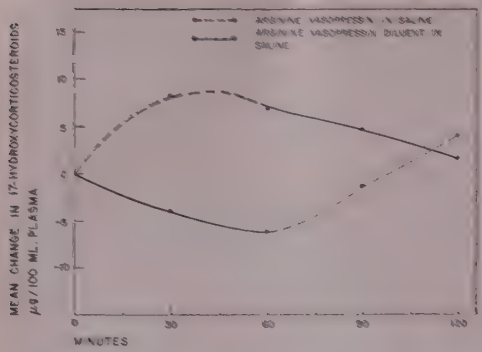

FIG. 1. Effect of arginine-vasopressin on plasma 17-hydroxycorticosteroid concentration. Individual points represent mean values for a group of 5 young, normal subjects receiving 12 to 16 units of arginine-vasopressin in saline in one-hr infusion either preceded or followed by one-hr infusion of saline plus arginine-vasopressin diluent.

randomized in both groups. Blood samples were drawn immediately before, and 15, 30, and 60 minutes after injection.

Results. The pretest levels of plasma 17-OHCS based on the 34 initial values obtained in this study averaged 16.4 μg % with a standard deviation of 5 μg %.

Infusion of Arginine-Vasopressin. The effect of intravenous infusion of arginine-vasopressin is shown in Fig. 1. Plasma 17-OHCS levels associated with infusion of arginine-vasopressin are significantly higher than those associated with infusion of vasopressin diluent. Increases observed are comparable in degree to those associated with infusion of similar amounts of Pitressin. The degree of nausea, abdominal cramping, and peripheral vasoconstriction experienced by individuals receiving infused arginine-vasopressin was similar to that noted with infusion of Pitressin(1).

Intravenous injection of Pitressin and vasopressin diluent. Injection of identical volumes of Pitressin and vasopressin diluent was undertaken to determine their effects on plasma 17-OHCS. It is apparent from Fig. 2 that injection of 2 units of Pitressin causes a rise in plasma 17-OHCS, whereas no rise occurs with injection of vasopressin diluent. The rise associated with injection of Pitressin is significant at 30 minutes ($P<0.01$) and is probably significant at 15 and 60 minutes ($P< 0.05$).

Intravenous injection of arginine-vasopressin and lysine-vasopressin. Both arginine-vasopressin and lysine-vasopressin cause an increase in plasma 17-OHCS in this experimental situation. As shown in Fig. 2, the increase associated with intravenous injection of 2 units of lysine-vasopressin is more than twice that observed when 2 units of arginine-vasopressin are given in the same manner. The different degree of response following injection of these 2 substances is probably real ($P<0.02$ at 30 minutes; $P<0.05$ at 15 and 60 minutes).

Summary. (1) Intravenous administration of highly purified arginine-vasopressin in man is associated with a significant increase in plasma concentration of free 17-OHCS.

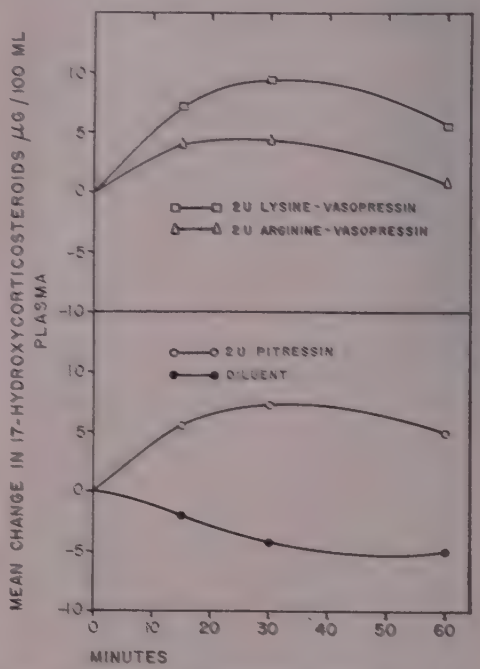

FIG. 2. Effect of intravenous injection of 2 U Pitressin, lysine-vasopressin, arginine-vasopressin, or equivalent vol of diluent, on plasma 17-OHCS concentrations. *Upper graph*, mean values of 8 young, normal males receiving, on different days, lysine-vasopressin and arginine-vasopressin. *Lower graph*, mean values of 4 young, normal males receiving, on different days, Pitressin and vasopressin diluent.

(2) Highly purified lysine-vasopressin causes higher plasma 17-OHCS levels than highly purified arginine-vasopressin when administered by means of an intravenous injection. (3) Increase in plasma concentration of free 17-OHCS observed when Pitressin is administered in man can be completely accounted for by the vasopressin content of Pitressin.

The authors wish to express appreciation to Daniel W. Clink, Francis A. Esposito, and Raymond W. Patrick for technical assistance.

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Nithiazide I. Chemical and Biological Studies on 1-ethyl-3-(5-nitro-2-thiazolyl) urea and Related Compounds. (22519)

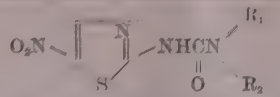
ASHTON C. CUCKLER, CHRISTINE M. MALANGA, ARTHUR J. BASSO, ROBERT C. O'NEILL AND KARL PFISTER, 3rd. (Introduced by H. J. Robinson.)

Merck Institute for Therapeutic Research and Merck, Sharp and Dohme Research Laboratories, Rahway, N. J.

Enterohepatitis (blackhead disease) may be an important cause of morbidity and mortality in turkey flocks. Although a number of substituted 8-hydroxyquinolines and arsenical compounds(1,2) were known to have some value in enterohepatitis, the discovery of the effectiveness of 2-amino-5-nitrothiazole by Waletzky *et al.*(3) revealed an important new chemical means of controlling this disease. During the course of screening various substituted ureas(4), we found that 1-ethyl-3-(5-nitro-2-thiazolyl) urea was effective in controlling the morbidity and mortality of enterohepatitis. This compound has been assigned the generic name of nithiazide. The present paper reports the results of the chemical and biological studies on nithiazide and related compounds.

Materials and methods. Chemical. The 1-mono-substituted nitrothiazolylureas employed in this study were prepared by the addition of various isocyanates to 2-amino-5-nitrothiazole. The preparation of 1-ethyl-3-(5-nitro-2-thiazolyl) urea (Example 1) is representative of this general method. Synthesis of 1,1-disubstituted nitrothiazolylureas was conveniently effected by heating 2-amino-5-nitrothiazole and a disubstituted carbamyl chloride in an inert solvent (Example 2). The basicity of 2-amino-5-nitrothiazole is sufficiently feeble that the hydrogen chloride produced in this reaction is readily expelled by the refluxing solvent. *Example 1. 1-Ethyl-3-(5-nitro-2-thiazolyl) urea.* To a solution of 69 g (0.97 mole) of ethyl isocyanate and 1200 ml of dry toluene was added 94 g (0.648 mole) of 2-amino-5-nitrothiazole. The stirred suspension was heated 16 hours at vigorous reflux under scrupulously anhydrous conditions. The warm slurry (80°) was filtered, and the pale yellow crystalline cake of 1 - ethyl - 3-(5 - nitro - 2 - thiazolyl) urea was washed well with benzene and ether, and then dried to constant weight. Yield: 137.7 g (98.5%); m.p. 228° (dec.) Anal. Calcd. for $C_6H_6O_3N_4S$: C, 33.33; H, 3.73; N, 25.91. Found: C, 33.70; H, 3.58; N, 25.44. Infrared spectrum (Nujol mull): Intense carbonyl band at 5.95 μ; broad N-H absorption at 3.2 μ. Ultraviolet spectrum (ethanolic hydrochloric acid): $\lambda_{max.}$ 350 mμ $\epsilon_{max.}$ 14910; $\lambda_{max.}$ 234 mμ, $\epsilon_{max.}$ 7890. *Example 2. 1,1-*

TABLE I. Comparative Antihistomonad Activity of 1-Substituted-3-(5-nitro-2-thiazolyl) Ureas.



1-substituted-3-(5-nitro-2-thiazolyl) ureas

Substituent	R_1	R_2	Melting point	Relative antihistomonad activity (%) compared to 1-ethyl compound
1-methyl	H	CH_3	243.5° (dec.)	75
1,1-dimethyl	CH_3	CH_3	164–5°	25
1-ethyl	H	C_2H_5	228° (dec.)	100
1-isopropyl	H	$CH(CH_3)_2$	230–231° (dec.)	25
1-n-butyl	H	C_4H_9	200–202°	50
1-n-octyl	H	$n\text{-}C_8H_{17}$	156–7°	<10
1-n-octadecyl	H	$n\text{-}C_{18}H_{37}$	143–4°	<10
1-phenyl	H	C_6H_5	228° (dec.)	<10
1-p-nitrophenyl	H	$p\text{-}C_6H_4NO_2$	256° (dec.)	<10

Dimethyl-3-(5-nitro-2-thiazolyl) urea. A solution of 14.5 g (0.10 mole) of 2-amino-5-nitrothiazole and 16.1 g (0.15 mole) of dimethylcarbamyl chloride in ethylene dichloride (100 ml) was stirred at reflux for 2½ hours. On cooling the amber solution to room temperature, a heavy deposit of crystalline 1, 1 - dimethyl - 3 - (5 - nitro-2-thiazolyl) urea was obtained. The product was filtered, washed well with ether and dried. Yield: 21.3 g (98.5%); m.p. 164-5°. Anal. Calcd. for $C_6H_8O_3N_4S$: C, 33.33; H, 3.73. Found: C, 33.51; H, 3.90.

Biological. Experimental enterohepatitis was produced in young turkeys with cultures of *Histomonas meleagridis* as described in the second paper in this series. The 1-substituted-3-(5-nitro-2-thiazolyl) ureas were incorporated in the ration in concentrations of 0.1% and fed to the infected turkeys beginning on the third day after inoculation. The drug diets were fed until the 14th day after infection. The relative activity of the compounds was determined by their effect on preventing mortality and eradicating cecal and hepatic lesions of enterohepatitis. The effect of nithiazide on experimental trichomoniasis (*Trichomonas foetus*), trypanosomiasis (*Trypanosoma brucei*), coccidiosis (*Eimeria tenella*) and malaria (*Plasmodium gallinaceum*) was determined by standardized methods with appropriate reference standards for comparison(5).

Results. The comparative antihistomonad activities of nine 1-substituted-3-(5-nitro-2-thiazolyl) ureas are summarized in Table I. These data show that among the mono-substituted compounds the most effective were the 1-methyl and 1-ethyl derivatives. Increasing the length of this substituent beyond the butyl derivative decreased the activity of the compounds. Compounds with 8 or more carbon atoms in this substituent were relatively ineffective. The 1-methyl compound was slightly less effective and considerably more toxic (growth retardation) than the 1-ethyl derivative. The dimethyl substituted compound was less effective and less toxic than the mono-methyl compound. The 1-ethyl substituted compound was the most effective compound in enterohepatitis and, therefore, was selected for further evaluation in other parasitic protozoan infections.

In experimental trichomoniasis in mice, nithiazide eradicated body cavity infections of *T. foetus* when administered intraperitoneally in a dosage of 100 mg per kg on the 5th, 6th and 7th days of the infection. Under these conditions, nithiazide was equally as potent as aminitrozole, 2-acetylamino-5-nitrothiazole(5). However, when administered orally, nithiazide was less effective than aminitrozole. Nithiazide did not exhibit antimalarial, antitrypanosomal or anticoccidial

activity under appropriate standardized conditions of evaluation.

Summary. 1. The preparation of 1-monosubstituted and 1,1-disubstituted nitrothiazolylureas has been described. 2. Antihistomonad activity was demonstrated for monosubstituted thiazolylureas with not more than 4 carbon atoms. The 1-methyl compound was more toxic and less effective than the 1-ethyl derivative. The dimethyl compound was less toxic and also less effective than the mono-methyl compound. 3. In addition to therapeutic activity in enterohepatitis, nithiazide [1-ethyl-3-(5-nitro-2-thiazolyl) urea] was found to have antitrichomonal activity but antimalarial, antitrypanosomal or anticoccidial activity was not demonstrable.

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Nithiazide II. Effect on Enterohepatitis in Turkeys. (22520)

ASHTON C. CUCKLER AND CHRISTINE M. MALANGA. (Introduced by H. J. Robinson.)
Merck Institute for Therapeutic Research, Rahway, N. J.

Earlier studies(1) showed that nithiazide, 1-ethyl-3-(5-nitro-2-thiazolyl) urea, was an effective chemotherapeutic agent for histomoniasis and trichomoniasis, but ineffective for certain other parasitic protozoan infections. The present paper reports the results of studies on the effect of nithiazide on experimental enterohepatitis (blackhead disease) in turkeys.

Materials and methods. Day-old straight run Beltsville white poults were obtained from a commercial source. The poults were reared in electrically heated battery-brooders. They were fed an open-formula commercial turkey starter and water *ad lib.* When 2 weeks of age, they were sorted into balanced weight groups of 5 poults each. On the 15th or 16th day of age, the poults were inoculated intracloacally with 2 ml of a 48-hour culture suspension of *Histomonas meleagridis* grown in the Balamuth's(2) egg infusion medium. The number of histomonads in the inocula varied from 1 to 4 million per ml. In the various experiments, no attempt was made to adjust the number of organisms prior to inoculation since maximal infectivity and mortality were wanted. In the experiment in which enterohepatitis was induced with cecal worm infections, the poults were each inoculated orally with approximately 200 embryonated *Heterakis gallinae* ova. The groups of poults were then assigned randomly to unmedicated basal ration or to basal ration plus graded amounts of nithiazide or 2-amino-5-nitrothiazole. Graded concentrations of these compounds were thoroughly blended into turkey starter ration just prior to use. Nithiazide and the sodium salt of nithiazide, which was administered in graded concentrations in drinking water of the turkeys, were supplied by Merck Sharp and Dohme Research Laboratories. A total of 1267 poults were used in these studies. The graded levels of nithiazide required 707 poults whereas 480 poults served as untreated controls and 80 poults were utilized for comparative studies with 2-amino-5-nitrothiazole. The experiments in which poults were inoculated with *Histomonas* culture were terminated on the 14th day after infection. The experiment in which *Heterakis* eggs were used to produce the *Histomonas* infection was terminated on the 21st day after inoculation of the poults because the infection from this source develops more slowly. The experiments designed to determine relapse rate were ended 21 days

TABLE I. Comparative Effect of Nithiazide and 2-amino-5-nitrothiazole in Experimental Enterohepatitis Produced with Cultures of *Histomonas meleagridis*.

Medication	% in feed	No. poults	Mean wt (g) Initial	Mean wt (g) Terminal	% infection	Mean lesion score Ceca	Mean lesion score Liver	% mortality
Controls	.00	256	209	341	91	3.4	3.0	73
Nithiazide	.0125	65	213	368	86	3.2	.4	35
	.025	119	218	445	43	1.2	0	4
	.05	115	209	466	7	.07	0	0
	.1	59	217	466	2	.03	0	0
2-amino-5-nitro-thiazole	.025	30	192	361	73	2.4	1.1	23
	.05	20	181	373	30	.6	0	0
	.1	30	194	342	3	.1	0	0

after withdrawal of medicated ration. In all experimental turkeys, enterohepatitis was demonstrated as the cause of mortality and the surviving turkeys were sacrificed and autopsy examinations were made for pathological lesions characteristic of infectious enterohepatitis. The presence and severity of the lesions in ceca and/or livers were scored as follows: 0 = normal, 1 = minimal detectable and 4 = maximal. Other criteria used for assessing the extent of disease in untreated control and treated experimental turkeys included incidence of infection, mortality rate and relative growth rates.

Results. The data obtained with graded concentrations of nithiazide in the feed are summarized in Table I. The non-medicated control turkeys had an infection rate of 91% and the mortality rate was 73%. In addition, control poults had well-developed cecal and hepatic lesions of enterohepatitis and survivors had markedly depressed rates of growth. A dose-response effect was observed with the various levels of nithiazide medication. The feeding of 0.0125% nithiazide to turkeys, starting 3 days after exposure to infection and continuing for 11 days, had little effect on decreasing percentage of birds infected. However, the mortality rate was reduced to slightly less than 50% of that in controls. The severity of liver lesions was also significantly reduced by feeding a diet containing 0.0125% nithiazide. The infectivity and mortality rates were further decreased when the level of medication in feed was increased to 0.025% nithiazide. When this dosage was used, cecal lesion scores were significantly less than in untreated control poults and none of the 119 nithiazide-fed poults examined had characteristic lesions of hepatic histomoniasis. There was no mortality in turkeys fed 0.05% or 0.1% nithiazide and cecal lesions were present in 7% and 2%, respectively. In most instances these lesions were very small; they may have represented the residual of partially healed lesions. No attempt was made to demonstrate the presence of viable histomonads in these lesions.

Comparatively, the results with graded concentrations of 2-amino-5-nitrothiazole have shown that nithiazide was approximately twice as potent as the former compound. In addition to greater therapeutic potency, nithiazide also was significantly better tolerated than 2-amino-5-nitrothiazole.

The effect of nithiazide on enterohepatitis induced in turkeys by inoculation of cecal

TABLE II. Effect of Nithiazide on Enterohepatitis Induced with *Heterakis gallinae* Ova Administered Orally.

% nithiazide in feed	No. poults	Mean wt (g) Initial	Mean wt (g) Terminal	% infection	Mean lesion score Ceca	Mean lesion score Liver	% mortality
.00	18	154	344	100	4	3.6	83
.0125	18	155	528	67	2	1.1	0
.025	18	155	589	61	1.2	.5	0
.05	17	150	616	0	0	0	0

TABLE III. Effect of Nithiazide Administered in Feed or Water on Mortality and Relapse in Experimental Enterohepatitis.

Relation of start of medication to infection	Days of medication	% in feed or water	No. poults	Enterohepatitis mortality (%) During medication	Enterohepatitis mortality (%) After medication	Enterohepatitis mortality (%) Total
1 day before	21	.025 feed	58	2	3	5
3 days after	18	.025	57	11	8	19
Controls	—	None	59	—	—	86
5 days after	9	.025 feed	40	25	37	62
Idem	"	.05	40	0	37	37
Controls	—	None	39	—	—	85
7 days after	14	.1 feed	12	—	—	8
Idem	"	.2	11	—	—	0
Controls	—	None	12	—	—	50
3 days after	18	.005 water	5	—	—	40
Idem	"	.01	20	—	—	0
"	"	.02	5	—	—	20
"	"	.04	10	—	—	0
7 days after	14	.01	10	—	—	0
Idem	"	.04	18	—	—	11
Controls	—	None	56	—	—	73
10 days after	11	.01 water	5	—	—	40
Idem	"	.04	5	—	—	0
Controls	—	None	20	—	—	100

worm (*Heterakis gallinae*) ova is presented in Table II. In this experiment treatment was started 3 days after infection and continued for 18 days. The results show that feed concentrations of 0.0125% and 0.025% nithiazide markedly reduced the incidence of infection and severity of intestinal and hepatic lesions. Nithiazide in a feed concentration of 0.05% was completely effective in eradicating all evidence of infection and in preventing mortality from enterohepatitis. The greatest weight gains were made by poults fed 0.05% nithiazide.

The data shown in Table III were obtained to determine the influence and relationship of time of initiation and duration of medication on cure and relapse rates in enterohepatitis. When 0.025% nithiazide was fed starting 1 day before or 3 days after infection and continued for 18 or 21 days, mortality from enterohepatitis was significantly reduced. During a 21-day period of observation after withdrawal of medication, relapses were observed in 3% of poults fed nithiazide prophylactically. In the therapeutic trial started 3 days after infection, a relapse rate of 8% was observed. When treatment was begun 5 days after infection and continued for only 9 days, the relapse rate was 37% for each of the groups of poults fed 0.025% and 0.05% nithiazide. When dosage of nithiazide was increased to 0.1% or 0.2%, treatment was highly effective even when delayed until the 7th day after infection. Although clinical symptoms and mortality were observed in untreated control poults on the 7th day after inoculation, the use of nithiazide at this time, in concentrations of 0.1% or 0.2% in feed or 0.01% or 0.04% in water, significantly reduced or completely prevented mortality from enterohepatitis. Furthermore, administration of 0.04% nithiazide in water beginning on the 10th day after infection prevented mortality from an infection of enterohepatitis which was lethal for all untreated control birds.

Discussion. The data presented show that nithiazide is highly effective for prevention or treatment of enterohepatitis in turkeys. Although nithiazide (1-ethyl-3-(5-nitro-2-thiazolyl) urea) is related to 2-amino-5-nitrothiazole, the potency and toxicity of the compounds differ markedly(3,4). Nithiazide was approximately twice as potent as 2-amino-5-nitrothiazole for controlling mortality from enterohepatitis. The growth

of turkeys fed 0.1% 2-amino-5-nitrothiazole for only 2 weeks was greatly retarded whereas nithiazide in a feed concentration of 0.1% had little effect on growth of young turkeys.

The feeding of nithiazide at 0.025% concentration in the ration 1 day before or 3 days after infection with *H. meleagridis* was highly effective in preventing mortality from enterohepatitis. Furthermore, the feeding of 0.05% nithiazide 3 days after infection was completely effective in preventing mortality from infectious enterohepatitis which killed 73% to 83% of untreated controls.

When treatment was not started until 5 days after infection and continued for only 9 days, nithiazide in feed concentration of 0.025% was relatively ineffective. Under these conditions, a feed concentration of 0.05% was effective in preventing mortality during medication; however, the 9-day period of medication was insufficient and relapses occurred in 37% of the poults. When the concentration of nithiazide was increased to 0.1% or 0.2% and the period of feeding was extended to 14 days, mortality from enterohepatitis was effectively controlled even when medication was delayed until the 7th day after infection. The use of 0.01% to 0.04% nithiazide in the water, beginning 3 days or 7 days after infection, effectively prevented mortality from enterohepatitis. Mortality from enterohepatitis was also prevented with 0.04% nithiazide administered in the water 10 days after infection.

Summary. 1. The data presented show that nithiazide [1-ethyl-3-(5-nitro-2-thiazolyl) urea] was more potent and less toxic than 2-amino-5-nitrothiazole when fed to turkeys with enterohepatitis. 2. Nithiazide was most effective when administered prior to infection or beginning 3 to 7 days after infection. It prevented mortality from enterohepatitis produced either by oral exposure to cecal worm eggs or by rectal inoculation of *Histomonas meleagridis* cultures. Nithiazide may be administered in the feed or water and is equally effective in either form. 3. The therapeutic feeding of rations containing 0.05% nithiazide, starting 3 days after infection, prevented mortality from infectious enterohepatitis which was lethal for 73% to 83% of the untreated control turkeys.

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Inhibition of Experimental Nephrocalcinosis by Hypophysectomy.* (22521)

H. SELYE, P. BOIS, AND J. VENTURA.

Institut de Médecine et de Chirurgie expérimentales, Université de Montréal, Montreal, Canada.

Excessive administration of various mono- or dibasic phosphate solutions causes extensive renal calcification in rats. In unadrenalectomized animals (sensitized for the nephrotoxic action of mineralocorticoids by unilateral nephrectomy), this nephrocalcinosis is aggravated by desoxycorticosterone acetate (DOCA) and inhibited by cortisol acetate (COLA)(1-3). From this it was concluded that the corticoids exert an important regulating influence upon the ability of the kidney to handle an excess of phosphate. Yet nephrocalcinosis can be induced by phosphate even after complete adrenalectomy(4). The question now arose whether pituitary hormones are necessary for induction of nephrocalcinosis by combined treatment with DOCA and an excess of phosphate. This appeared of interest, because nephrocalcinosis is so markedly enhanced by DOCA, a steroid whose other nephrotoxic actions (the produc-

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TABLE I. Inhibition of Experimental Nephrocalcinosis by Hypophysectomy.

Group	No. of rats	Treatment	Renal lesions: Nephrocalcinosis	Renal lesions: Hyalin casts	Renal lesions: Cortical atrophy
1	10	Intact, $Na_2H\ PO_4$ p.o.	Severe (only in zona intermedia)	None	None
2	17	Hyp-X, " "	None	"	"
3	10	Intact, NaH_2PO_4 s.c.	Severe (throughout cortex)	Numerous	Intense
4	17	Hyp-X, " "	Trace	None	"

tion of malignant nephrosclerosis and proteinuria) are virtually abolished by hypophysectomy(5,6).

Materials and methods. Fifty-four female Sprague-Dawley rats, having an average initial body-weight of 101 g (range: 92-111 g), were subdivided into 4 groups and treated as shown in Table I. The hypophysectomies in Groups 2 and 4 were performed through the parapharyngeal approach. On the day of the operation and on the following day all animals received 1 mg of COLA† subcutaneously, while 100 μg of DOCA† per day were administered in the same manner to the animals of all 4 groups throughout the experiment. The glucocorticoid was given during the immediate post-operative period only in order to raise the resistance of the hypophysectomized rats, but for uniformity all groups were similarly treated. On the other hand, since previous experiments had shown that glucorticoids inhibit nephrocalcinosis, only DOCA (which facilitates the production of this change) was administered during the rest of the observation period.

In Groups 1 and 2 an aqueous 1% Na_2HPO_4 solution was made available *ad lib*, as a drinking fluid. This salt is more effective in producing nephrocalcinosis than NaH_2PO_4, but also more irritating to tissues and, therefore, it does not lend itself as well to subcutaneous administration. Groups 3 and 4 were kept on ordinary tap water and received NaH_2PO_4 in the form of a 5% solution subcutaneously twice daily, the individual dose being gradually raised from 1 to 5 ml during the first 5 days and then maintained on the same level. Normally, it is impossible to administer such high concentrations and volumes of NaH_2PO_4, because multiple subcutaneous hemorrhages and skin-necroses ensue. We therefore used the "granuloma-pouch" technic(7), which makes it possible to give highly irritating solutions parenterally without causing significant local tissue-damage. For the purpose of the present experiment 25 ml of air was injected subcutaneously under the shaved skin of the back, this being immediately followed by the introduction of 1 ml of 5% NaH_2PO_4 into the air sac thus formed. The resulting local inflammation transforms the lining of the air sac into a thin but resistant granuloma which withstands the increasing concentrations of phosphate solution and yet permits salt absorption into the bloodstream. In this manner we could inject a total of 10 ml of 5% NaH_2PO_4 daily between the fifth and the tenth day after hypophysectomy. The experiment was terminated on the 11th day by killing the animals with chloroform. Immediately after autopsy one kidney was fixed in neutral formalin (for the histochemical demonstration of calcium deposits with Kossa's silver nitrate technic) and the other in Susa solution (for subsequent staining with the Periodic Acid Schiff, or "PAS," stain, to demonstrate hyalin, fibrinoid material).

Results. Upon naked-eye inspection of the cut surface, a chalky line of *calcium deposition* was clearly visible between the cortex and medulla of the kidney, in the "zona intermedia" [terminology of Heidenhain(8)], in all the rats of Group 1. The kidneys in Group 3 showed a more diffuse white discoloration—especially of their surface underneath the capsule—but no obvious change could be detected in Groups 2 and 4.

Table I lists the mean degree of nephrocalcinosis (assessed in a scale of 0 to

† The authors are indebted to Pfizer Laboratories for generous supplies of cortisol acetate ("Cortril") and to Schering Corp. for desoxycorticosterone acetate ("Cortate").

+++), as judged by microscopic observation of tissues stained with the Kossa technic. It should be added that even histochemically virtually no calcification could be detected in the outer cortex of the animals of Group 1, although marked calcification occurred in the zona intermedia of the kidney, where it stimulated the secondary formation of granulomatissue with giant cells. In Group 3, calcium deposits were seen in the proximal convoluted

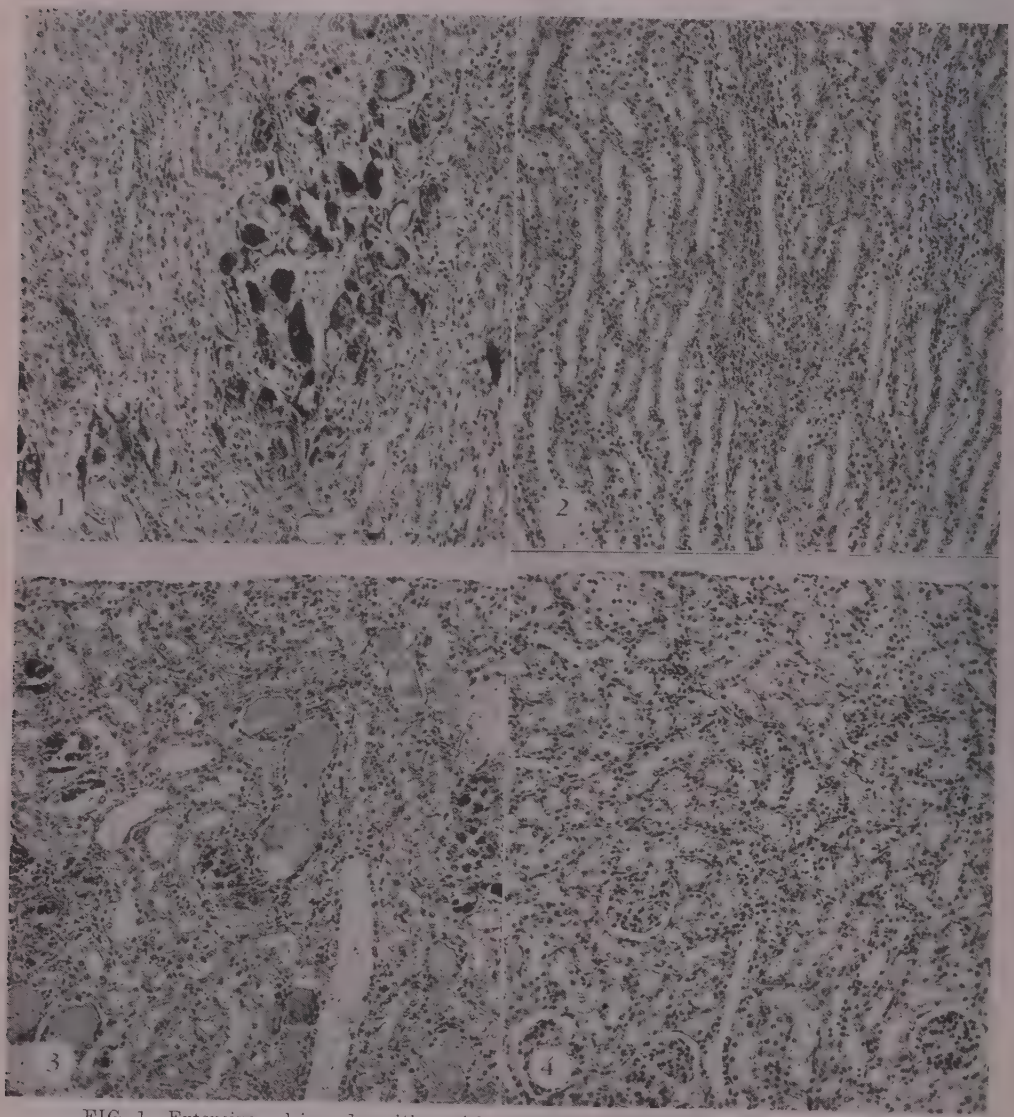

FIG. 1. Extensive calcium deposition with reactive inflammation and giant-cell formation in the "zona intermedia" of the kidney of a rat from Group 1 (Kossa stain × 100).

FIG. 2. "Zona intermedia" of the kidney of a rat from Group 2. Calcium deposition was completely prevented by hypophysectomy (Kossa stain × 100).

FIG. 3. Subcapsular renal cortex of an intact rat from Group 3. Diffuse calcium deposition and hyalin-cast formation (Kossa stain × 100).

FIG. 4. Subcapsular renal cortex of a rat from Group 4. Hypophysectomy completely prevented both calcium deposition and hyalin-cast formation (Kossa stain × 100).

tubules throughout the entire width of the renal cortex; they were particularly numerous in the subcapsular region. On the other hand, in the hypophysectomized rats of Group 2, which drank phosphate solution, virtually no calcium deposits could be demonstrated, except for one rat, in which hypophysectomy proved to be incomplete. Among the hypophysectomized rats of Group 4, given phosphate solution subcutaneously, only 6 survived until the end of the experiment. Two of these showed very slight degrees of diffuse nephrocalcinosis, although no remnants of pituitary tissue could be detected at autopsy. All 15 remaining animals in this group (whether or not they survived until termination of the experiment) were free of detectable calcium deposits (Fig. 1-4).

Hyalin casts and precipitated proteinaceous material were plentiful in the renal tubules of the rats of Group 3, but were absent in all other groups.

A very unusual change in the structure of the kidneys was found in Groups 3 and 4. Here a marked *selective atrophy of the outer renal cortex* had developed under the influence of the subcutaneously administered phosphate. The epithelial cells of the proximal convoluted tubules became unusually small and their brush-border assumed an irregular lumpy appearance or disappeared completely. The cytoplasm tended to lose its staining ability and, in many cases, contained fine, hyalin, PAS-positive droplets. The nuclei in the proximal tubules did not appear to participate in this atrophy so that, in many nephrons, the entire lining looked somewhat like a "macula densa," consisting of aggregates of many nuclei, with but little, poorly staining, cytoplasm. The basement membranes around the atrophic tubules were thickened and strongly PAS-positive. Curiously, the capillary vessels throughout this involuting outer cortex were greatly dilated (Fig. 5-9).

Discussion. It is evident that, under our experimental conditions, in intact rats treated with DOCA the administration of phosphate in the drinking water produced the usual nephrocalcinosis, limited to the zona intermedia of the kidney, while subcutaneously introduced phosphate caused a more diffuse calcification of all parts of the kidney and especially of its subcapsular outer cortex. It is true that, in this particular experiment, Na_2HPO_4 was given *per os* and NaH_2PO_4 subcutaneously, but this could not account for the observed difference. Our earlier experiments on nephrocalcinosis had repeatedly shown that both the mono- and the dibasic phosphates cause calcification predominantly in the zona intermedia of the kidney (without cortical atrophy), when administered by mouth, and in the outer cortex (with cortical atrophy), when injected into the granuloma-pouch subcutaneously. Perhaps, in the latter event, the sudden flooding of the organism with large concentrations of phosphate is responsible for the diffuse renal damage, while when only moderate amounts are taken in gradually (*per os*), the nephron-segments specifically designed for the handling of excess phosphate are more selectively affected. In any event, it is clear that hypophysectomy greatly inhibits—and indeed usually abolishes—the nephrocalcinosis due to excess phosphate, irrespective of whether the subcutaneous or the oral route of administration is employed. This cannot be due merely to a decreased phosphate absorption. Daily measurement of the fluid-intake in Groups 1 and 2 showed it to be essentially the same in the intact and the hypophysectomized rats (usually about 35 ml/day). Furthermore, in Groups 3 and 4, at autopsy only an average of 5-8.8 ml of phosphate solution could be recovered from the granuloma-pouches (which corresponds to less than the amount injected in a single day), and chemical analyses invariably revealed a dilution, rather than a concentration, of phosphate in solutions thus introduced into subcutaneous air sacs.

In view of the fact that the hyalinosis of the kidney normally produced by DOCA-overdosage is prevented by hypophysectomy (5,6), it is interesting that the hyalin-cast formation, which resulted from the subcutaneous injection of phosphate, was likewise prevented in the absence of the pituitary (*cf.* Groups 3 and 4). On the other hand, the in-

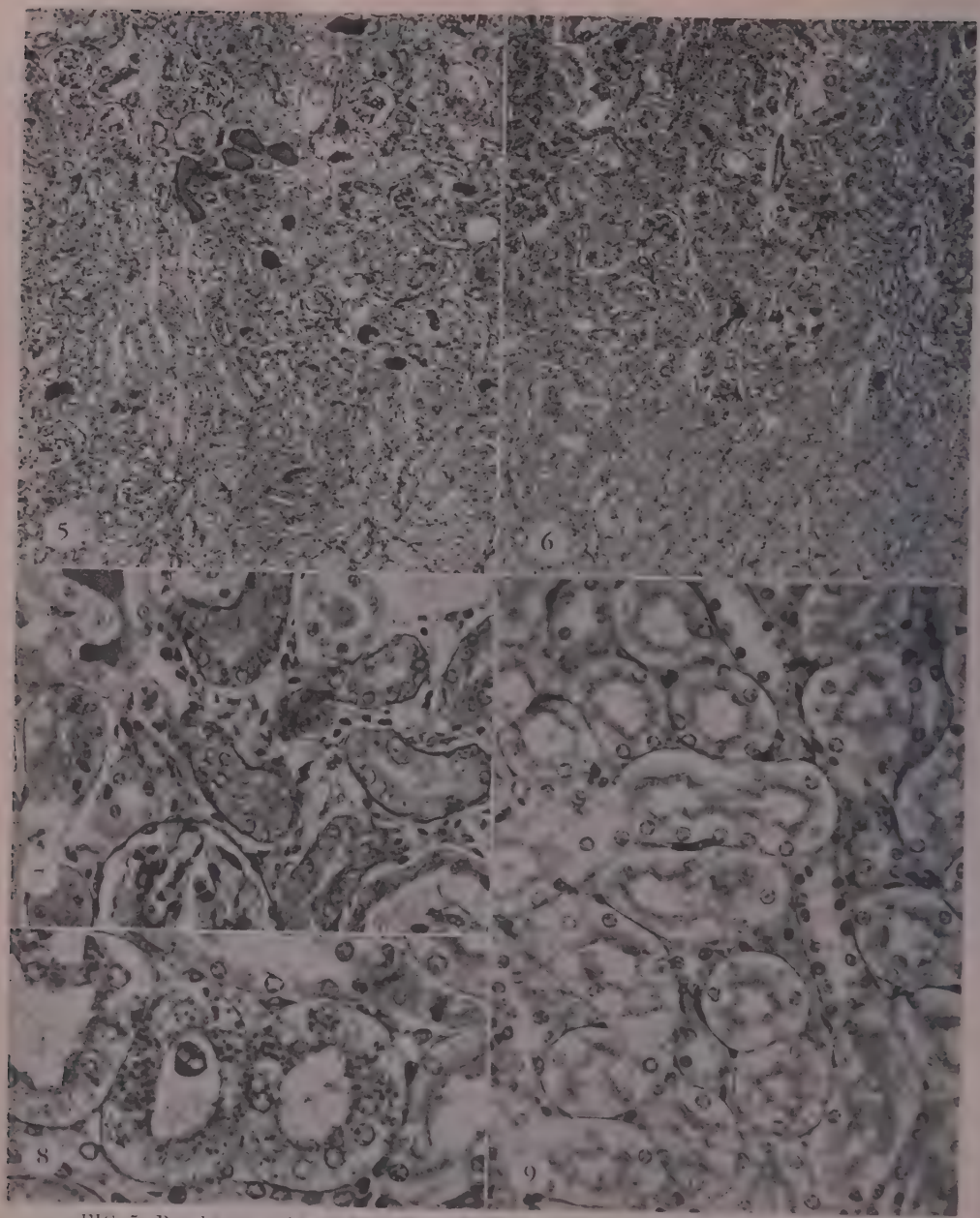

FIG. 5. Renal cortex of a rat from Group 3. The outer region, which contains the glomeruli, has undergone marked atrophy and disorganization of its pattern, due to connective-tissue proliferation. Many tubules are obstructed by dense casts, which are intensely stained with the Periodic Acid Schiff reagent (PAS stain × 75).

FIG. 6. Renal cortex of a rat from Group 4. Hypophysectomy has completely prevented hyalin-cast formation, but not the induction of cortical atrophy (PAS stain × 75).

FIG. 7. Representative field from the kidney shown in Fig. 5. Note atrophy of the tubules, thickening of basement membranes and proliferation of connective tissue, which appears to compress the tubules. Because of selective involution of the cytoplasm and virtual disappear-

ance of cell borders, "macula-densa-like" apparently syncytial structures arise, in which the nuclei are often actually enlarged and, in some places, deformed (PAS stain × 400).

FIG. 8. Higher magnification of another region of the kidney shown in Fig. 5. Here the PAS-positive, small, hyalin granules within the tubular epithelium are clearly visible. Note also that the brush-border tends to accumulate in clumps or is almost completely shed off (PAS stain × 720).

FIG. 9. Proximal convoluted tubules from the inner, not atrophic, stratum of the cortex shown in Fig. 5, 7 and 8. Unlike in the region shown in Fig. 7 (which is photographed at the same magnification), the tubules in this stratum (chiefly composed of spiral segments) are normal, the brush-border is well developed, the cytoplasm abundant and the stroma hardly detectable (PAS stain × 400).

tense and rather selective atrophy of the outer cortex was equally evident in the intact and in the hypophysectomized rats receiving subcutaneous NaH_2PO_4. Presumably, this change, unlike the nephrocalcinosis and the hyalin-cast formation, is not significantly dependent upon any of the pituitary hormones.

Summary. 1) Experiments on rats indicate that oral administration of excess sodium phosphate causes intense calcification in the "zona intermedia" of the kidney in intact but not in hypophysectomized rats. 2) The "granuloma-pouch" technic permits the subcutaneous administration of as much as 10 ml of 5% NaH_2PO_4 per rat daily. (Normally, this amount could not be given subcutaneously because of its topical necrotizing effect.) When so administered, the phosphate solution causes a generalized nephrocalcinosis throughout the kidney and this is also virtually abolished by hypophysectomy. Apparently, hypophyseal hormones play an important conditioning rôle in the pathogenesis of this kind of nephrocalcinosis. 3) Repeated injections of hypertonic phosphate solutions, into subcutaneous granuloma-pouches, cause the formation of numerous hyalin casts and protein-precipitates in the kidney and this change was likewise totally abolished by hypophysectomy. When thus administered, phosphate solutions can also induce a pronounced and highly selective atrophy of the entire outer renal cortex, but this change is essentially of the same severity in the presence and in the absence of the hypophysis.

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Mechanism of Heparin Protection Against a Histamine Releasor (48 80).* (22522)

R. D. HIGGINBOTHAM AND THOMAS F. DOUGHERTY.†

Department of Anatomy, University of Utah College of Medicine.

It was previously reported that fibroblasts ingest a variety of metachromatic substances which are deposited in their cytoplasm in granular forms(1,2). Since the recognition of mast cells is dependent primarily on a specific (metachromatic) staining of their cytoplasmic granules, rather than any definitive shape or appearance of the cell(3), the granulated fibroblasts are analogous to tissue mast

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cells and may be described as "quasi-mast cells" to indicate this superficial resemblance. It was postulated that micellophagosis, *i.e.*, the fibroblastic ingestion of mucopolysaccharides, may play an important role in local detoxification by the absorption and intracellular degradation of conjugates of (tissue) mucopolysaccharides with noxious agents(2). When the conjugate is metachromatic and initially deposited in the cytoplasm as granules, this intermediate phase of detoxification would be characterized by the appearance of fibroblasts having metachromatic granules in their cytoplasms.

The phenyl alkyalmine, 48/80, has been found to induce acute intoxication in laboratory animals following parenteral injection (4), as well as definite cytological changes in the mast cells of the loose connective tissue (5). It was of interest to investigate the possibility that the acidic mucopolysaccharides of ground substance could modify the toxicity of this compound by complexing with the amine and thereby initiating micellophagosis of the toxic agent.

Methods. Protection study. Mice 2 to 3 months old and ranging in weight from 21 to 25 g were challenged intraperitoneally with various doses of 48/80‡ contained in a volume of 0.25 ml. Both C-57 and CBA mice were used in this study since no marked differences in their respective reactions to the 48/80 were noted. A 48/80 LD_{50} of 4.0 $\pm$ 0.3 μg/g of mouse was established. Additional mice were pretreated with various amounts of heparin (117 I.U./mg)‡ injected in a volume of 0.25 ml by the intraperitoneal route. Thirty minutes later these animals were challenged with standard doses of 48/80 and the survivals noted. Fifty to 115 mice were used per 48/80 challenge group and the amount of heparin necessary to protect 50% of the mice against the respective challenge doses of 48/80 was calculated by probit analysis. The mucopolysaccharides, chondroitin sulfate, and hyaluronic acid were prepared as saline solutions for comparison in activity with heparin. *Tissue Study.* CBA mice, 8 to 10 weeks old, were injected subcutaneously on the dorsal surface with 1.0 cc of air to form an air pouch in the loose subcutaneous connective tissue. Test substances, in a volume of 0.2 cc, were injected into the bubble formed within the loose subcutaneous connective tissue. At the appropriate time the animals were sacrificed and the connective tissue bubble dissected free of the surrounding tissues. The ventral surface of it was exposed and a portion removed, spread on a glass microscope slide, air-dried, and stained with May Gruenwald-Giemsa stain in the usual manner(6). Similar samples were removed and stained with Azure A for comparison. Test substances were suspended in isotonic saline and injected in 0.2 cc volumes containing a) 500 μg heparin, b) 100 μg 48/80, c) a mixture of 500 μg heparin and 100 μg 48/80, and d) a saline control. Ten animals were used for each group and were sacrificed 30 minutes following injection and tissue samples were obtained. The tissues were examined with the aid of oil immersion magnification, and the following types of cells counted: a) agranular fibroblasts, b) fibroblasts with a few cytoplasmic granules, and c) cells, some of which were of uncertain origin, whose cytoplasm contained numerous metachromatic granules. No distinction was made between intact and degranulating mast cells. Five hundred cell counts per tissue spread for each of the 10 samples of the 4 groups were performed.

Results and observations. Preliminary observations showed that the 50% lethal dose of 48/80 varied markedly with the route of injection (Table I). For any one route, mice

TABLE I. Effect of Injection Route and Treatment on Toxicity of 48/80.

Variation in 48/80 toxicity relative to inj. route		Effect of some mucopolysaccharides on 48/80* toxicity	
Route	LD_{50}, μg/g	Polysac. (200 μg)	Survivors
Subcut.	17.6	Hyaluronic acid	2/20
Intraper.	4.0	Chondroitin sulfate	0/20
Intrav.	1.5	Heparin	19/20
		Saline	0/20

* 7.2 μg/g, i.p.

‡ The authors wish to express their appreciation to Dr. Edgar A. Jack of Burroughs-Wellcome & Co. and to Dr. L. L. Coleman of Upjohn Co. for their generous gifts of 48/80 and heparin, respectively.

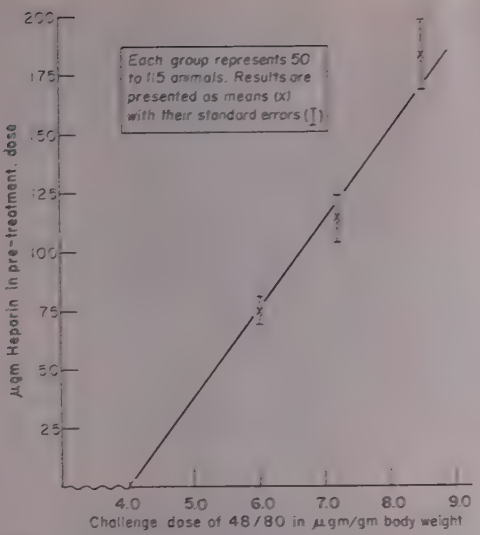

FIG. 1. Dose response curve illustrating protective effect of heparin pretreatment in mice against subsequent challenge with varying amounts of 48/80 in which the ordinate represents the heparin dose sufficient to protect 50% of the mice against a given challenge dose of 48/80.

were separated into groups of 10 animals each and injected with graded doses of the amine contained in 0.25 ml saline. The numbers of survivors 24 hours later were noted and the LD_{50} calculated(7). The results as shown in Table I could be interpreted to indicate that tissue constituents may bind with the toxic amine and thereby account for the differences noted in its toxicity relative to the route of administration.

Groups of 10 mice each were then pretreated with intraperitoneal injections of saline containing 200 μg hyaluronic acid, chondroitin sulfate, or heparin, and challenged 30 minutes later with a lethal dose of 48/80 (7.2 μg/g). The experiment was repeated and the accumulated results, presented in Table I, indicated that only heparin offered a marked and (what has since been found to be) a reproducible type of protection against the toxic effects of the amine.

Protection. A quantitative study of the dose response relationship between heparin pretreatment doses and standard 48/80 challenge doses (6.0, 7.2 or 8.5 μg/g) was then performed. All of these 48/80 doses were rapidly lethal to non-heparinized (saline) control mice. The results in terms of the 50% protective doses of heparin were plotted against their respective 48 80 challenge doses, as shown in Fig. 1. The straight line, obtained by the method of least squares, suggests that the increased tolerance of the heparin-treated mice may be due to a direct interaction of these two drugs *in vivo*. When solutions of the two drugs are mixed together, a cloudy solution results. Mota, *et al.*(5) have reported 48/80 inhibition of heparin anticoagulant activity *in vitro*.

Fibroblastic granulopoiesis. Experiments were then performed to determine the cellular responses involved in this heparin-enhanced tolerance to 48 80. It was noted that in mice pretreated with 500 μg of heparin intravenously and then injected subcutaneously with 100 μg 48 80, the fibroblasts in the injection area often had numerous small metachromatic granules in their cytoplasm, which suggested that cellular mechanisms were involved in the enhanced tolerance of heparin-treated mice to 48 80. When a mixture of heparin and the amine was injected, many of the cells were found to contain metachromatic granules in their cytoplasms. An experiment was then carried out to determine the requirements for this granulopoietic response. Four groups of animals were used as noted above. In the saline controls (Fig. 2, 3) the pouching and injection procedures caused a slight degranulation of some mast cells with a subsequent uptake of the shed granules by nearby fibroblasts(8) (Table II) during the 30-minute interval between time of injection and sampling of the tissue. The injection of heparin alone did not markedly alter the relative distribution of the cells, and the appearance of the tissue was essentially the same as those of the controls, except for the strong metachromasia of the ground substance due to the presence of the injected mucopolysaccharide. On the other hand, 48/80 caused a widespread disruption of mast cells (Fig. 4, 5), as previously reported(5). The increase in granulated fibroblasts (Table II) appeared to be partially due to the increased number of shed mast cell granules

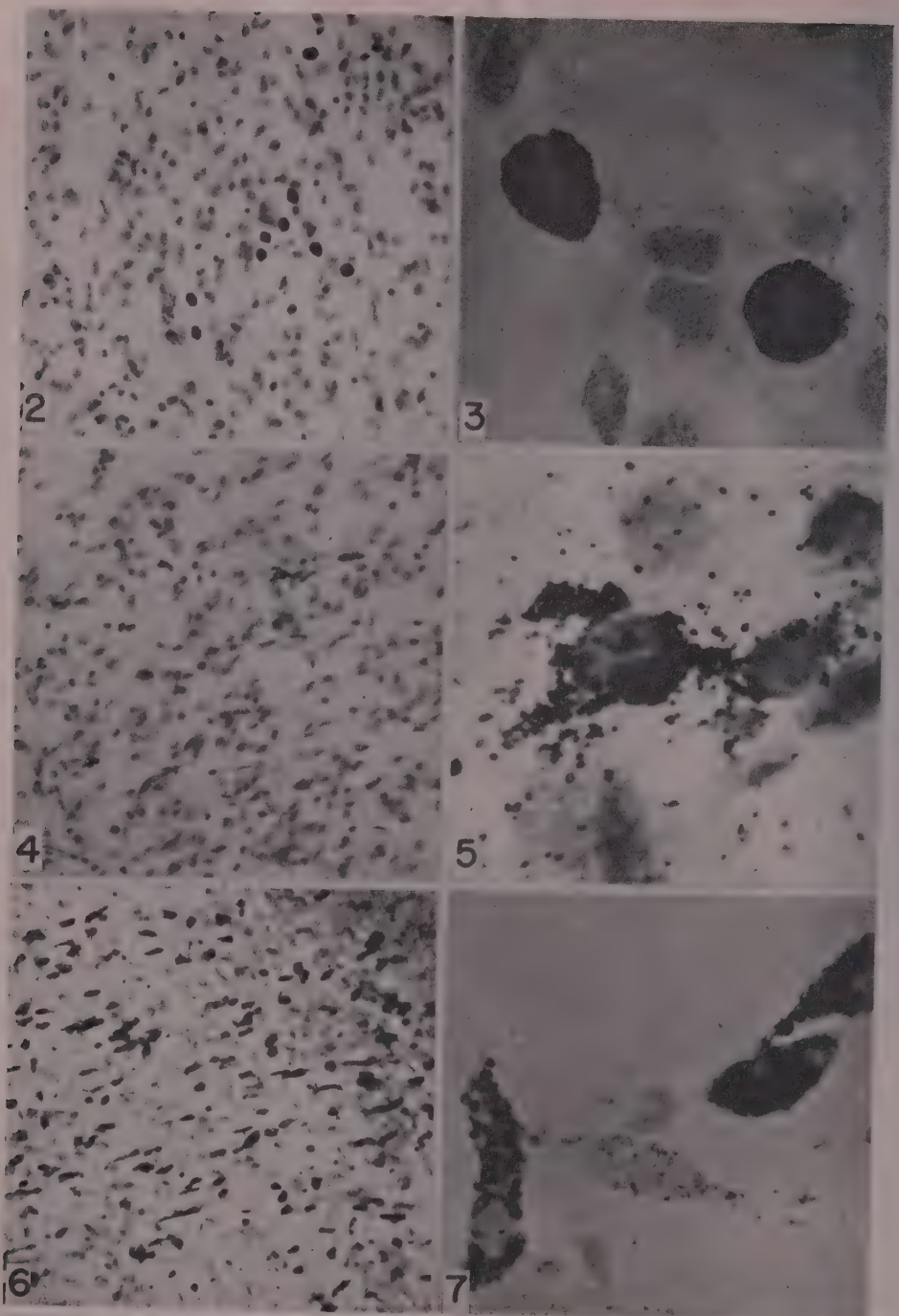

FIG. 2-7. Air-dried spreads of mouse loose connective tissue obtained 30 min. after local injection of test materials. Stained with May Gruenwald-Giemsa stain.

FIG. 2. Saline control tissue (× 150).

FIG. 3. Same as Fig. 2 with increased magnification showing intact mast cells of control tissue (× 1550).

FIG. 4. Tissue after 48/80 injection. Note widespread degranulation of mast cells (× 150).

FIG. 5. Same as Fig. 4 with increased magnification showing mast cell response to 48/80 injection (× 1550).

FIG. 6. Tissue after 48/80-heparin injection. Note increased concentration of cells exhibiting dense metachromatic cytoplasms as compared to Fig. 2 and 4 (× 150).

FIG. 7. Same as Fig. 6 with increased magnification showing the cytoplasmic granulation of fibroblasts in response to treatment. Note fibroblasts in center with few granules, in contrast to heavily granulated cells in lower left and upper right of field (× 1550).

available for fibroblastic uptake(8). The tissues from animals injected with the 48/80-heparin complex contained an increased concentration of cells with metachromatic granules in their cytoplasms (Fig. 6, Table II). Fig. 7 shows the morphology of some of these cells with the cytoplasmic granulation induced by heparin-48/80 treatment. The granules in some cells were coarse as in mast cells; in some they were rather fine, and in others there was a mixture of both types of granules. The elongated cytoplasm of many of these quasi-mast cells contrasts markedly with the rounded-up appearance of the typical connective tissue mast cell in the mouse. However, variant morphological types are not infrequently observed in this animal.

It is obvious from Fig. 6 that a local injection of the heparin-48/80 complex will induce an increased *concentration* of metachromatically granulated cells in the loose connective tissue. The *percentage distribution* of these cells in the tissue, as well as their general morphology, suggests that they originated from agranular fibroblasts (Table II).

TABLE II. Percentage Distribution* of Granulated Cells in Loose Connective Tissue following Subcutaneous Injection of Saline, Heparin, 48/80 or Heparin-48/80 Complex.

Treatment group	Fibroblasts	Sparsely granulated fibroblasts	Heavily granulated cells
	%		
Saline	90.1 ± 1.4 (87.2-93.0)	4.3 ± .6 (3.0-5.6)	5.7 ± 1.1 (3.4-7.9)
Heparin	87.0 ± 2.2 (82.2-91.7)	5.9 ± 1.0 (3.7-8.1)	7.1 ± 1.4 (4.2-10.0)
48/80	72.8 ± 2.0 (68.6-77.0)	21.1 ± 1.9 (17.0-25.2)	6.1 ± 1.2 (3.5-8.7)
Heparin-48/80	6.5 ± 1.8 (2.8-10.3)	16.4 ± 2.0 (12.2-20.6)	77.1 ± 2.9 (71.1-83.1)

* Results presented as percentage of means and stand. errors with 95% confidence limits on 500 cell counts per tissue spread. Nine to ten spreads examined for each group.

The fate of these granulated cells was obscured by the resultant inflammatory reaction to the injection material, but observations made at intervals suggested that much of the material was digested by the cells. Twenty-four hours after injection, the number of granulated cells, as well as the number of granules per cell, appeared to be markedly decreased.

Discussion. The ubiquitous distribution of loose connective tissue throughout the body as "packing material" of the blood vessels implies its importance as an intermediary in the transportation of metabolites to and from parenchymal cells. The presence of the macromolecular acidic mucopolysaccharides in the ground substance of this tissue suggests that it may have a physiological role as a "buffering" or detoxifying system for noxious amines, thereby enabling the tissue to maintain the local environment within a physiological range. This particular function of the acidic mucopolysaccharides could be analogous to actions of cation exchange resins.

The binding of the toxic phenyl alkylamine, 48/80, by heparin and its sequestration in granular form by fibroblasts of the tissue has been presented as a model of this local detoxifying response of the loose connective tissue. The beneficial results arising from experimentally increasing the tissue concentration of heparin prior to challenge with an otherwise lethal dose of 48/80 supports this concept. Unpublished results indicate that other sulfated polysaccharides such as dextran sulfate are also effective. Conversely, heparin treatment seems to enhance tolerance of mice to other noxious basic substances such as polymyxin B or stilbamidine(9).

Comparison of the granulated fibroblast to tissue mast cells would appear to be rather inopportune since knowledge concerning the

cellular precursor(s) as well as the function of the mast cells is largely speculative. However, certain similarities between these cells are apparent, as noted in the results. It is noteworthy that morphological similarity can be enhanced if the mice are treated with hydrocortisone before injection of the heparin-48/80 complex(9). The hormone treatment induces a rounding-up of the fibroblasts(6) and the quasi-mast cells produced from these cells are therefore without cytoplasmic processes.

Chemically, the similarity is limited to the presence of heparin in the granules of both types of cells(10), since the mast cell granule has been reported to contain also histamine (11), 5-OH tryptamine(12), chymotryptic activity(13), etc.(14,15), in contrast to the phenyl alkylamine content of the heparin-48/80 cell. However, granulated fibroblasts can also be induced by heparin complexes with other substances such as protamine (basic protein)(16), polymyxin B (basic polypeptide), or stilbamidine (diamidine)(9). In addition, numerous other metachromatic polysaccharides can be ingested by fibroblasts and give rise to cytoplasmic granules(9). It is of interest that injury to these cells can initiate a shedding of the granular material similar to mast cell degranulation(9).

From the results presented, it seems probable that metachromatically granulated cells may arise and persist for varying periods of time in the tissues as a result of fibroblastic uptake of material not readily digested by the cell. The presence or persistence of such material would be a function of the rates of cellular uptake and disposal which could be considered as relative to the metabolic state of the cell as well as to the "foreignness" of the ingested material. In this manner, tissue obtained from various pathological states may give the appearance of an increased concentration of "mast cells" largely as a result of present and past states of interaction between connective tissue cells and a noxious environment.

Summary. 1. Mice pretreated with various amounts of heparin exhibit resistance, relative to heparin dose, to the toxic effects of subsequent injections of otherwise lethal doses of 48/80. There is a straight-line relationship between the 50% protective heparin pretreatment doses and the various 48/80 challenge doses. 2. Fibroblasts exposed to the heparin-48/80 complex rapidly sequester and deposit this material as metachromatic granules in their cytoplasm. 3. The possible significance of these results is discussed.

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Variation in Sensitivity of *Escherichia coli* to Freezing Damage During the Growth Cycle.* (22523)

KOHEI TOYOKAWA† AND DAVID H. HOLLANDER. (Introduced by T. B. Turner.)
Department of Microbiology, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Md.

The degree of susceptibility of bacteria to freezing damage has been said to vary during the growth cycle. Fry and Greaves(1) noted better (15%) survival when a 20-hour culture of a paracolon bacillus was dried from the frozen state, than (3%) when a 4½-hour culture was frozen and dried. Similar experiences were recounted at the 1954 London Freeze-Drying Discussion(2).

This variation was examined in the following experiments.

Methods. The general methods employed were similar to those previously described(3). The organism was an old laboratory strain of *Escherichia coli.* Trypticase soy broth (BBL) was used for cultures and dilutions, and trypticase soy agar (BBL) for plate counts. Plate counts were made with tenfold serial dilutions. Freezings and thawings were accomplished with 2.0 ml quantities in 100 x 13 ml pyrex tubes in an alcohol dry ice bath and in a 35° water bath for 10 minutes each. Glycerol was not used in these experiments.

A. *Comparison of sensitivity of a 3-hour and a 24-hour culture of E. coli.* Aliquots of a 3-hour and of a 24-hour culture were subjected to from 1 to 10 cycles of freezing and thawings after which the survivors were determined by the plate counting method (Fig. 1). The cells of the 24-hour culture were more resistant and were destroyed at a slower rate during the repeated freezings and thawings.

B. Changes in sensitivity during growth cycle. Plate counts were made at hourly intervals from a 20-hour 40-ml culture of *E. coli.* Essentially logarithmic growth was observed during the first 5 hours (Table I). Aliquots taken at the same time were counted after 2 cycles of freezing and thawing (Table I). Freezing and thawing was much more destructive for the cells of the logarithmic growth phase, than for the cells of the original inoculum, or those after 5 hours of growth. In this experiment the amount of destruction caused by 2 cycles of freezing and thawing increased from about 75% with the original inoculum, to more than 99.9% after 1 hour, and then decreased after 5 hours.

Discussion. Many of the properties of bacterial populations vary during the growth cycle. This is true for cell morphology and staining(4,5); bacterial metabolism(6,7); susceptibility to injury by heat(8-10); cold (11); and phenol(10); susceptibility to damage by changes in osmotic pressure(12); and by changes in barometric pressure(13); and for susceptibility to injury by ionizing radiations(14).

It is of interest that the changes in susceptibility to freezing damage during the growth cycle are parallel with changes in susceptibility to osmotic pressure(12), and barometric pressure(13), since these pressures have been implicated in the mechanism of freezing damage. However, since the changes

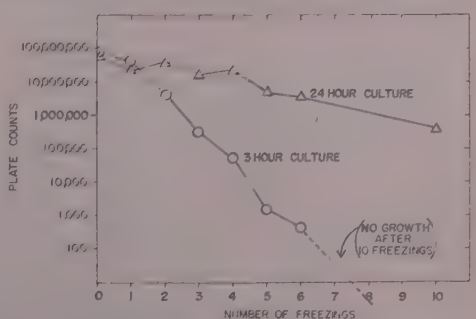

FIG. 1. Plate counts of *E. coli* cultures after successive freezings and thawings.

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† Present address—Department of Hygiene, School of Medicine, University of Tokyo, 1 Motofuzi, Hongo, Bunkyo-ku, Tokyo, Japan.

TABLE I. Plate Counts of an *Escherichia coli* Culture at Intervals during Growth, Unfrozen and after 2 Cycles of Freezing and Thawing.

Age of culture (hr)	Unfrozen	Frozen twice	Surviving fraction after freezings (%)
0	33,000	9,300	28.2
1	480,000	238	.05
2	2,500,000	1,320	.05
3	32,000,000	4,070	.01
4	183,000,000	102,000	.06
5	600,000,000	44,000,000	7.3
6	620,000,000	162,000,000	26.1
7	950,000,000	73,000,000	7.7
8	760,000,000	72,000,000	9.5
9	650,000,000	102,000,000	15.7

which occur during the growth cycle are poorly understood it does not seem profitable to speculate on these changes in relation to the mechanism of freezing damage.

Since it is apparent that the phase of growth may have a tremendous influence on the susceptibility of bacteria to freezing damage, it is important to consider this factor in the interpretation of freezing damage data.

Summary. The sensitivity of *Escherichia coli* to freezing damage varies during the growth cycle with maximum susceptibility during the period of logarithmic growth.

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Hydrolysis of Amino Acid Amide Derivatives of 2-Aminofluorene, 4-Aminobiphenyl and 4, 4′-Diaminobiphenyl by Leucine Aminopeptidase.* (22524)

ROBERT L. HILL† AND EMIL L. SMITH.

Laboratory for the Study of Hereditary and Metabolic Disorders, and Departments of Biological Chemistry and Medicine, University of Utah College of Medicine, Salt Lake City.

It has been established that carcinogenic amines, such as 2-aminofluorene, 4-aminobiphenyl and 4,4′-diaminobiphenyl (benzidine), combine *in vivo* with tissue proteins or protein precursors(1-3). Although the exact mode of combination with proteins is unknown, Greenstein, *et al.*(4) have suggested that the binding may involve a peptide bond between the amino group of the amine and a carboxyl group of the protein or protein precursor. Moreover, these investigators have demonstrated that peptide derivatives, in which the carcinogenic amine is linked to the carboxyl group of different amino acids, are susceptible to hydrolysis by tissue homogenates. Although the enzyme or group of enzymes responsible for the hydrolytic action has not been identified, it is reasonable to assume on the basis of

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† Public Health Service Research Fellow of the National Institute of Arthritis and Metabolic Diseases.

TABLE I. Rates of Hydrolysis of Certain Amino Acid Amides. 0.05 M substrate was incubated at 40°C for 4 hr in presence of 0.06 M Tris [tris(hydroxymethyl)aminomethane] buffer at pH 8.5, 0.002 M $MnCl_2$, 60% methanol and 1.3 γ protein N/ml of aminopeptidase. The enzyme was activated for 30 min. under the same conditions in absence of substrate(5). AF = 2-aminofluorene, BP = 4-aminobiphenyl, Bz = benzidine.

Substrate	C_1
L-Leucinamide	11.
L-Norvalyl-AF	.14
L-Isoleucyl-AF	.026
L-Valyl-AF	.015
L-Phenylalanyl-AF	.01
Glycyl-AF	.01
L-Norvalyl-BP	.135
D-Norvalyl-BP	0
L-Isoleucyl	.01
L-Valyl-BP	.01
L-Norleucyl-BP	.028
L-Tryptophyl-BP	.015
D-Norleucyl-BP	0
L-Norleucyl-AF*	+++++
L-Phenylalanyl-BP*	++
D-Phenylalanyl-BP*	0
Glycyl-BP*	++
Di-L-Alanyl-Bz*	+++
Di-Glycyl-Bz*	±

* These compounds were too insoluble to permit determination of an approximate C_1 value. Apparent extent of hydrolysis could be estimated qualitatively by observing amount of amino acid detected after chromatographic analysis of the reaction mixtures.

the known requirements for the specificity of leucine aminopeptidase(5), that this enzyme could hydrolyze the carcinogenic amine-peptide bond. All tissue homogenates used by Greenstein, *et al.*(4) are known to contain this enzyme(6). It is the purpose of this paper to present data on the hydrolysis of several amino acid amide derivatives of carcinogenic amines by leucine aminopeptidase.

Methods. The leucine aminopeptidase used in this study was prepared from swine kidney essentially as previously described(7). The derivatives of the carcinogenic amines were prepared by Dr. J. P. Greenstein and his associates(4). We wish to express our thanks to them for their courtesy and generosity in furnishing samples for this study.

Activation of the aminopeptidase was performed essentially as described earlier(5). However, the insolubility of most of the amino acid amide derivatives in water prevented studying rates of hydrolysis under the usual conditions. Since the compounds are more soluble in methanol, 60% methanol was used for study of the enzymatic reaction. The effect of methanol on the rate of hydrolysis of amino acid amides by the aminopeptidase has been studied(8).

Results. Table I shows that all of the L-isomers tested are hydrolyzed by the enzyme although the rates are slow when compared with the sensitive substrate, L-leucinamide. When possible, proteolytic coefficients (C_1) were determined for each of the substrates by the titrimetric procedure of Grassmann and Heyde(9) in order to obtain at least a rough comparison of the susceptibility of each amide to the aminopeptidase. (C_1 is the first order velocity constant k_1 per mg protein N per ml calculated in minutes and decimal logarithms). Like the unsubstituted amides, those compounds with aliphatic side chains are more susceptible to hydrolysis than those with aromatic side chains(5). However, some reservation should be made in considering the relative rates of hydrolysis, since the degree of solubility of each compound in 60% methanol is different and would influence the rate of hydrolysis. Furthermore, methanol does not inhibit hydrolysis of all amides to the same extent(8). L-Norvalylaminofluorene appears to be hydrolyzed at a greater rate than the L-norleucine derivative, which is unexpected on the basis of previous specificity studies(5). However, the norvaline compounds are completely soluble in 60% methanol whereas the norleucine compounds are only partly soluble. For the enzyme preparation used, the C_1 for L-leucinamide was 60 in water whereas it was 11 in 60% methanol. In order to confirm the titrimetric results shown in Table I, each reaction mixture was examined for free amino acids after completing the experiment. Excess substrate was removed from 0.75 ml of each reaction mixture by addition of an equal volume of water. The mixture was centrifuged, the supernatant solution acidified to pH 2 and treated with Dowex-50-X5 (20-40 mesh) ion exchange resin in the H^+ cycle for adsorption of the free amino acids(10). After washing the resin thoroughly, the amino acids were eluted with 5N NH_4OH and the eluates con-

TABLE II. Inhibition of Leucine Aminopeptidase by Benzidine, 4-Aminobiphenyl and 2-Aminofluorene. Each reaction mixture contained 0.05 M leucinamide, 0.06 M Tris buffer at pH 8.5, 0.002 M $MnCl_2$, 30% methanol, 0.29 γ protein N/ml of aminopeptidase, and inhibitor at the concentration indicated. The enzyme was activated under the same conditions for 30 min. in absence of substrate.

Inhibitor	Inhibition (%)			
	.001 M	.005 M	.01 M	.05 M
Benzidine	5	8	22	30
4-Aminobiphenyl	20	20	23	25
2-Aminofluorene	6	—	14	25

centrated to dryness *in vacuo* over concentrated sulfuric acid. The eluted amino acids were then chromatographed on Whatman No. 1 filter paper in butanol-acetic acid-water (200-30-75) for 48 hours. The papers were dried and sprayed with a ninhydrin solution. The chromatograms showed only the amino acid present in the original amide derivatives. No free amino acids could be detected in control reaction mixtures incubated without aminopeptidase under the same conditions.

Further confirmation for enzymatic hydrolysis was obtained from the green-blue color which developed on addition of the reaction mixtures to the ion-exchange resin. Because of the oxidation of Mn^{++} to MnO_2 in the reaction mixture during hydrolysis, as well as the presence of free amine, conditions were favorable for the oxidation of the amines to benzidine blue and related compounds(11). Control reaction mixtures did not give any color nor did those compounds containing the D-isomers, which were resistant to enzymatic hydrolysis. Qualitatively, it appeared that those compounds hydrolyzed most rapidly also produced the most color. Under proper conditions it might be feasible to use this reaction for a quantitative estimation of free carcinogenic amine.

Since product inhibition of the aminopeptidase by the carcinogenic amines could influence the hydrolysis, each amine was tested for its inhibitory properties. The results are shown in Table II. It is seen that only at high concentrations of amine was there any significant inhibition. It is probable that any inhibition caused by free amine did not significantly affect the observed rates of hydrolysis, although the inhibition might account for the fall in velocity constants observed in some cases.

Discussion. The observations of Greenstein, *et al.*(4) have demonstrated that amino acid amide derivatives of certain carcinogenic amines are hydrolyzed by the enzymes of a number of animal tissues. The present study shows that a highly purified peptidase, namely leucine aminopeptidase, can catalyze the hydrolysis of such compounds. Although the present results do not exclude the possibility that other tissue enzymes may also catalyze this reaction, it is obviously unnecessary to assume the presence of novel enzymes to explain the hydrolysis of the amino acid amide derivatives of the carcinogens. Unfortunately, it is difficult to obtain a strictly quantitative comparison of the sensitivity of these compounds to the aminopeptidase, largely because of their insolubility in water. The results obtained in 60% methanol yield only approximations both of the absolute and relative rates of hydrolysis. Studies, to be published elsewhere(8), have demonstrated that the inhibition of the aminopeptidase by methanol and other alcohols varies with different substrates, the greatest degree of inhibition being obtained with the most sensitive compounds. For example, although Table I indicates that the L-norvalyl derivative of aminofluorene is hydrolyzed 14 times more rapidly than the glycyl analog, it is very likely that they differ in susceptibility by a factor which is at least 10 times greater. Nevertheless, the present results are in general accord with the known specificity of the enzyme, derivatives of the large aliphatic amino acids being more sensitive than the aromatic ones or the small aliphatic ones, glycine and alanine. In addition, derivatives of the D amino acids are completely resistant to the action of the enzyme, in agreement with many earlier studies(5,6).

Summary. Amino acid amide derivatives of the carcinogenic amines, 2-aminofluorene, 4-aminobiphenyl and 4,4′-diaminobiphenyl (benzidine) are hydrolyzed by highly purified leucine aminopeptidase of swine kidney. Because of the poor solubility of these com-

pounds in water, the studies were performed in 60% methanol. The results are in general accord with the known specificity of the aminopeptidase.

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Sequential Assays of 4 Amino Acids in Grains of Wheat, Rye, and of Their Hybrid.* (22525)

SIDNEY W. FOX† AND DOROTHY DE FONTAINE.
Department of Chemistry, Iowa State College, Ames.

The chemical characterization of organisms in ways that may be related to their biological evolution may be taken as a primary objective of comparative biochemistry. A review of peptide analysis(1) listed characterization of proteins for biological differentiation as a principal discernible goal, along with elucidation of amino acid residue sequence. Qualities of an ideal N-terminal agent were also prescribed. The agent with many of these attributes has proved to be fluorodinitrobenzene (FDNB), which has been successfully applied to problems of structure(2). FDNB has received somewhat less use in characterization(3). It has become apparent that a more powerful mode of characterization might be obtained from a method employing quantitative sequential analysis. It therefore proved to be desirable to employ phenylisothiocyanate(4) subtractively(5). Many data have been accumulated by this procedure. A hybrid of wheat and rye(6) afforded an opportunity to investigate chemical characterization as related to biological type. For this purpose, subtractive quantitative sequential peptide analysis was applied to seeds of wheat-rye hybrid (*Triticale*) and to its parents.

For the grains of corn, wheat, and rye, most amino acids have been found not to differ significantly in total content or in proportion in N-terminal and N-penultimate positions (7). As wheat and rye seeds, however, were significantly different in their quantitative contents of total and N-terminal lysine, lysine was chosen for simultaneous assay in wheat, rye, and the hybrid. Assays of leucine were also performed as a basis of comparison inasmuch as the differences in leucine content appeared to be significant. It was furthermore of interest to check on the C-terminality of leucine. Inasmuch as lysozyme is known to contain N-lysine(8,5) and C-leucine(9,10), and the cereals contain N-lysine(7), such analyses permit comparison of whole cereal protein with lysozyme. Methionine and phenylalanine were also studied.

Materials and methods. The parent strains were *Triticum aestivum* and *Secale cereale.* The hybrid, *Triticale,* was generously sup-

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† Present address: Oceanographic Institute, The Florida State University, Tallahassee.

TABLE I. Total, N-Terminal, N-Penultimate, and C-Terminal Contents of 4 Amino Acids in Wheat, Rye, and in Their Hybrid.

N content (%) (MicroKjeldahl)	Wheat (2.45) AT_0	Wheat (2.45) AT_1	Wheat (2.45) AT_2	Wheat (2.45) CT_1	Rye (1.82) AT_0	Rye (1.82) AT_1	Rye (1.82) AT_2	Rye (1.82) CT_1	Lysozyme standard
Leucine N/Total N (%)	*4.3 ± .2*	*4.1 ± .3*	*4.3 ± .1*		*3.9 ± .2*	*3.9 ± .2*	*3.8 ± .3*		6.9 ± .2
	4.3 ± .1	4.3 ± .1			4.0 ± .1	4.1 ± .1			6.5 ± .2
					4.1 ± .1				7.0 ± .2
			4.2 ± .2				3.8 ± .2		7.1 ± .2
				4.1 ± .1				*3.7 ± .1*	6.7 ± .1
				4.1 ± .1				*3.8 ± .1*	7.0 ± .1
Lysine N/Total N (%)	*3.0 ± .1*	*.79± .08*	*1.11± .13*		*4.4 ± .2*	*1.2 ± .1*	*1.46± .08*		5.6 ± .2
	2.9 ± .2			*2.8 ± .2*				*4.0 ± .2*	5.9 ± .2
			1.11± .09				1.82± .22		5.4 ± .2
	2.9 ± .2	1.0 ± .1			4.2 ± .1	1.3 ± .1			5.7 ± .3
Methionine N/Total N (%)	*.69± .02*	*.70± .02*	*.69± .02*		*.72± .02*	*.72± .02*	*.70± .03*		1.77± .06
	.87± .03	.78± .03			.70± .01	.81± .06			1.9 ± .06
	.88± .02				.84± .02				1.87± .07
			.78± .04				.70± .04		1.86± .08
Phenylalanine N/Total N (%)	2.3 ± .1	2.5 ± .05			2.3 ± .1	2.2 ± .1			3.2 ± .1
			2.5 ± .1				2.2 ± .1		3.3 ± .1

N content (%) (MicroKjeldahl)	Wheat × rye Triticale 1.96 AT_0	AT_1	AT_2	CT_1	Lysozyme standard
Leucine N/Total N (%)	*4.6 ± .2*	*4.5 ± .2*	*4.5 ± .2*		6.9 ± .2
	4.3 ± .1	*4.4 ± .1*	*4.3 ± .2*	*4.1 ± .1*	6.7 ± .1
	4.5 ± .2			*4.2 ± .1*	7.0 ± .1
Lysine N/Total N (%)	*3.5 ± .1*	*1.04 ± .07*	*1.26 ± .11*		5.6 ± .2
	3.3 ± .2		*1.05 ± .11*	*3.4 ± .1*	5.9 ± .2
Methionine N/Total N (%)	*.72 ± .02*	*.70 ± .03*	*.69 ± .04*	*.73 ± .02*	1.77 ± .06
Phenylalanine N/Total N (%)	2.5 ± .1	2.3 ± .1	2.3 ± .1	2.4 ± .1	3.1 ± .1

AT_n = Aminoid treatment followed by complete hydrolysis. CT_1 = One carboxoid treatment followed by complete hydrolysis.

plied by Dr. J. G. O'Mara of the Genetics Department. The seeds were ground in a Wiley mill to pass a No. 40 mesh sieve. Moisture contents of the wheat-rye hybrid and its wheat and rye parents were 8.4, 9.1, and 8.7% respectively.

The italicized figures (Table I) comprise values obtained from the hybrid and from its parents. Results on any one line were obtained in a simultaneous set of assays. The non-italicized values represent a non-parental strain of wheat (Rival) and a non-parental strain of rye (spring rye).

The methods have been mostly described (5). Hydrolyses were conducted in excess 3 N hydrochloric acid in an autoclave at 15 lb pressure for 16 hr. No significant differences in results were found when redistilled hydrochloric acid was used under these conditions. Each hydrolyzate was filtered at pH 4 as recommended by Horn, Blum, Gersdorff, and Warren(11).

Assays were controlled by simultaneous assay of the same amino acid in an hydrolyzate of Armour crystalline lysozyme (Lot No. 003L1) with aliquots of the same organism and same medium. The standard deviations (Table I) are calculated from the estimations performed in quadruplicate at each of 5 levels.

The critical subtractive lysine assay method has been shown to be applicable to an N-lysine protein [lysozyme(5)] and to peptides with interior lysine [ACTH(12), bacitracin hydrolyzates(13)]. The use of such methods on unfractionated proteins is further justified by recent results in studies of chymotryptic proteolysis of lysozyme. The present type of analysis on unfractionated mixtures of peptides from proteolysis indicates that 2 of 3 phenylalanine residues in lysozyme are opened at their carbonyl end and that 2 of 3 tyrosine residues in the lysozyme molecule are opened at their carbonyl end by chymotrypsin(14). In a study of aromatic peptides isolated individually from the action of chymotrypsin on lysozyme, Acher, Laurila and Fromageot(15), employing several techniques of structural assignment on isolated peptides, also found 2 of 3 phenylalanine C-residues and 2 of 3 tyrosine C-residues opened by chymotrypsin. Aminoid residues opened by chymotrypsin, as reported in both types of study, comprise those of arginine, aspartic acid (asparagine), glycine, and isoleucine.

Results. The results are presented in Table I. No significant difference is found between each of the 2 strains of either wheat or rye. Significant amounts of free or N-terminal amino acids of those reported cannot be present except in the case of lysine, inasmuch as the other 3 amino acids show after one aminoid treatment a decrement which is less than experimental variation. Lysine also cannot be present in the free state inasmuch as the value following C-terminal treatment is the same as that for the untreated material.

Discussion. Wheat, rye, and wheat-rye hybrid all contain N-lysine. In this respect, they are closely related to corn, soy, and 2 blue-green algae. They differ from one of the known N-lysine proteins, lysozyme, in lacking valine in the N-penultimate position (5). They now appear to differ further from lysozyme in lacking C-leucine(9, Table I). The value of a quantitative subtractive N-terminal method for characterization of unfractionated proteins is demonstrated by these results. Conventional tagging procedures following fragmentation of unfractionated proteins would not permit conclusions on the sequential location of each type of amino acid.

The results with leucine indicate that the content of this amino acid in the hybrid is not intermediate between those of the parents. This relationship applies for the total content or that in the N-terminal position or in the N-penultimate position. The total lysine contents and the terminal lysine contents are, however, intermediate for the hybrid.‡ This result suggests that some amino acids, and/or some amino acids in particular positions in the protein portions of the organism, are more intimately involved with biological specificity than others§. Whether or not these

‡ The total N value is also intermediate.

§ This finding supports a similar earlier interpretation of the importance of basic amino acids, *cf.* Block, R. J., *Yale J. Biol. Med.*, 1935, v7, 235.

contents are fundamentally part of the genotype or part of the phenotype is of interest.

The answer to this question also bears on whether protein or nucleic acid is the heart of the genetic apparatus. Evidence can be cited for the importance of either type of structure (16,17). It is, therefore, possible that either protein or nucleic acid or both may function as ultimate determinants of biological specificity, or both may be manifestations of a more fundamental structure, or perhaps, interactions of structures. The present results, like those of Knight's study on tobacco mosaic virus proteins(16), indicate that amino acid-containing structures play a central role in some of such determinations. On the other hand, the limited diversity of types found in cereal seeds(7) by these criteria, as well as statistical analyses of amino acid composition(18), suggest that no very elaborate structural code would be required for the nucleic acids to determine types of protein.

Summary. Wheat, rye, and a wheat-rye hybrid have been analyzed for leucine, lysine, methionine, and phenylalanine. The assays have included total, N-terminal, N-penultimate, and C-terminal proportions of these amino acids. In all cases, only lysine is N-terminal. C-Terminal assays permit the conclusion that this lysine is all terminal, and not free. The proportions of total lysine and N-lysine in the hybrid are intermediate between the values in the parent strains. The comparable result for leucine is not intermediate.

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Latent Viral Infection of Cells in Tissue Culture. III. Role of Certain Amino Acids.* (22526)

ALFRED D. HEGGIE AND HERBERT R. MORGAN.
Louis A. Wehle Virus Research Laboratory, Department of Bacteriology, The University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

Previous investigations have demonstrated that tissue cultures of minced chick embryos, maintained for 11 to 14 days in a simple medium of inorganic salts and glucose, can be infected readily with psittacosis virus but do not support the multiplication of this agent (1,2). Following inoculation, such a latent infection can be maintained successfully for periods up to 15 days, the duration of cell survival under such conditions(2). Moreover, it has been established that viral multiplication can be induced at any time during this period by the addition of embryo extracts or

* This investigation was supported by a research grant (C-2223) from the National Cancer Institute, National Institutes of Health, Public Health Service.

by a synthetic tissue culture medium(1-3). The finding that the initiation of viral multiplication can be accomplished by the addition of a completely synthetic medium made it possible to attempt the definition of the specific stimulatory principles involved. Further work has established that a modification of Parker's synthetic tissue culture medium #199, *i.e.*, medium #635 (equivalent to #199 except for the elimination of the purines, pyrimidines, pentose sugars, adenosine, adenylic acid, and ATP; and the addition of increased amounts of cysteine, glutathione, and ascorbic acid) was as potent as medium #199 in stimulating viral multiplication(3). Essentially, medium #635 consists of amino acids, water and fat soluble vitamins, plus inorganic salts and glucose. Partial fractionation of this mixture was performed and the important stimulatory groups therein were found to be the amino acids and the water soluble vitamins(3).

The studies to be described were directed toward determining which of the component amino acids of medium #635 were important in initiating the multiplication of latent psittacosis virus in chick embryo cells.

Materials and methods. The virus studied was the 6BC strain of psittacosis virus, obtained originally from Dr. K. F. Meyer, and since passed repeatedly in eggs via the yolk sac route. The preparation of a uniform virus suspension has been described(2). A dilution of the standard virus suspension in Hanks's(4) balanced salt solution (BSS) to a concentration of $10^{2.0}$ to $10^{3.0}$ LD_{50} per ml was used to infect the tissue cultures.

Tissue cultures. As described previously (1), 10-day chick embryos were harvested and minced, and this minced tissue was washed 4 times with BSS containing 0.125 ml of a 1.4% solution of sodium bicarbonate per ml. Then aliquots of tissue were transferred to 10 ml Erlenmeyer flasks each containing a disc of perforated cellophane and 1.8 ml of BSS with 0.025 ml of 1.4% sodium bicarbonate. All cultures were incubated at 36°C. Culture fluids were changed completely after the first 24 hours and every 4 days thereafter. On the 13th day of incubation, the cultures were inoculated with psittacosis virus, and the virus inoculum was titrated(1). On the 14th and 18th days culture fluids were removed completely for titration and were replaced with equal amounts of a test medium, or in the control flasks with the complete stimulating medium or BSS. Fluids were removed and titrated a third time at the end of the experiment on the 22nd day. Virus titers in the culture fluids were calculated as $\log_{10}$ of the LD_{50} for 7 day embryonated eggs, according to the method of Golub(5). After removal of the culture fluids at the conclusion of each experiment, the remaining cells were fixed by the addition of a 1:1 ether-alcohol mixture, and the cellophane discs from various representative flasks were removed and stained by a modification of the Papanicolaou technic (6). The stained cellophane discs were examined microscopically to ascertain the quality and quantity of the cell population and to establish the presence or absence of psittacosis inclusion bodies.

Materials tested. All media tested were variations of Parker's medium #199(7) and were prepared according to the directions supplied by Dr. Parker, with the exception of cysteine which was used in a concentration of 100 mg per ml. The basic test medium, designated hereafter as the complete medium, contained the following constituents of Parker's medium #199: the amino acid and water soluble vitamin fractions, cysteine, inorganic salts and glucose. All fractions were prepared in stock solutions which were stored in the cold with the exception of tyrosine and cysteine which were stored at room temperature. The test media, each deficient in one amino acid, were prepared by appropriate combination of the stock solutions, and were sterilized by filtration through a sintered glass filter immediately before use. The sterile solutions were stored under refrigeration.

Experimental. These investigations were begun with the information that the water soluble vitamins and amino acids of Parker's synthetic medium #635 were capable of stimulating the growth of psittacosis virus in chick embryo tissue culture(3). Since it had previously been noted that the amino acid ana-

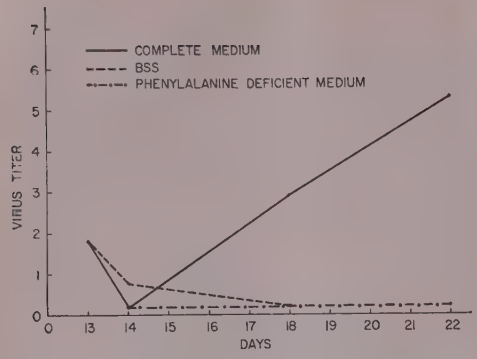

FIG. 1. Effect of phenylalanine deficiency on stimulation of growth of psittacosis virus (6BC) in chick embryo tissue cultures cultivated in BSS for 13 days.

logues, B-2-thienylalanine and 6-methyltryptophane, inhibited the growth of psittacosis virus in tissue culture(8), it was decided to test the effect of the omission of single amino acids on the stimulatory capacity of the complete medium. Accordingly, media were prepared which were identical to the complete medium in every respect except for the exclusion of one amino acid. In a series of experiments the stimulatory capacity of each of these deficient media was compared with that of the complete medium and with Hanks's BSS. It was found on repeated observations that phenylalanine and tryptophane were essential to stimulation of viral multiplication. Representative experiments are illustrated graphically in Fig. 1 and 2. Aspartic acid, hydroxyproline and lysine, on the other hand, were found not to be essential (Table I). At the conclusion of each experiment the cellophane discs from representative flasks were stained and examined as a check on cellular growth. In all cases, including those cultures grown in deficient media, viable cells were seen.

Discussion. The present studies confirm the finding that chick embryo cells grown *in vitro* in a deficient medium lose the capacity to support proliferation of psittacosis virus, but that this capacity can be restored by addition of certain nutrient substances to the culture fluids(1,2). It had already been shown that water soluble vitamins and amino acids alone could produce this same effect(3) and now it has been found that at least 2 amino acids are essential to stimulation of viral proliferation, while at least 3 are not. The finding that phenylalanine and tryptophane are essential agrees with the previous observation(8) that the corresponding amino acid analogues, B-2-thienylalanine and 6-methyl-tryptophane, inhibit the growth of psittacosis virus in tissue culture without showing evidence of toxicity for the host cells. Furthermore, it is of interest to note that these 2 amino acids are among those found by Eagle to be essential for the cultivation of mammalian cells *in vitro*(9).

The data presented lend additional support to the concept that the capacity of a cell to support the multiplication of a virus is directly related to the availability of essential nutrient substances to such a cell. Moreover, it would appear that viral multiplication is dependent not only upon an adequate supply of essential metabolites to the host cell, but that indeed a cell must be capable of a high level of metabolism if it is to support the proliferation of a virus. The mere viability of a cell by no means insures viral growth, for, as shown in the studies described herein, such growth never occurred in the absence of phenylalanine or tryptophane, although in every case viable cells were available to the virus. It is probable that the cell, even in this depleted state, has available sufficient quantities of essential metabolites to remain viable, but insufficient in amount to permit shunting of

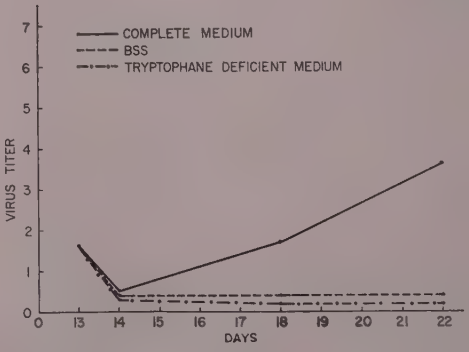

FIG. 2. Effect of tryptophane deficiency on stimulation of growth of psittacosis virus (6BC) in chick embryo tissue cultures cultivated in BSS for 13 days.

TABLE I. Effect of Various Amino Acid Deficiencies on Stimulation of Growth of Psittacosis Virus (6BC) in Chick Embryo Tissue Cultures Cultivated in BSS for 13 Days.

Amino acid deficiency		Virus titers*			
		13th day	14th day	18th day	22nd day
Aspartic acid		1.9	.1	4.7	5.0
Controls	Complete medium	1.9	.1	3.0	5.3
	BSS	1.9	.9	.1	.1
Hydroxyproline		3.1	.5	1.9	4.2
Controls	Complete medium	3.1	.1	2.1	4.2
	BSS	3.1	.1	.1	.1
Lysine		1.9	.1	3.9	5.4
Controls	Complete medium	1.9	.1	3.0	5.4
	BSS	1.9	.9	.1	.1

* Log of LD_{50}.

cellular metabolism into the reduplication of virus particles. Hence, when the virus enters a deficient cell, it remains present in the latent state in the non-infectious phase(2) until such time as the necessary metabolites become available, or until the death of the cell. In psittacosis, a disease characterized by long periods of latent infection in birds, this concept is of special interest, as a variety of changes in the host may lead to the activation of infection. As indicated by these studies, changes in cell nutrition as it relates to the amino acid may be important determinants in such activation.

Summary. Studies on the amino acid requirements for the activation of latent infection with psittacosis virus of chick embryo tissues cultivated *in vitro* revealed that phenylalanine and tryptophane are essential to the proliferation of psittacosis virus, while aspartic acid, hydroxyproline, and lysine are not. The importance of host cell metabolism to viral infection is discussed with special reference to the amino acids.

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Tungstate Antagonism of Molybdate in *Aspergillus niger*.* (22527)

EDWIN S. HIGGINS,† DAN A. RICHERT AND W. W. WESTERFELD.
Department of Biochemistry, State University of New York, College of Medicine at Syracuse.

A requirement for molybdenum by *Aspergillus niger* has been well established(1), particularly when nitrate is the sole source of nitrogen(2). Mo is specifically required in the nutrient medium for the enzymatic reduction of nitrate(3), and Nicholas and Nason (4) showed that it is part of the prosthetic group of the enzyme nitrate reductase. With Mo present in the growth medium, no nitrogen compounds other than the nitrate ion are

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† Present address: Department of Biochemistry, Medical College of Virginia, Richmond.

TABLE I. Tungstate Inhibition of Growth of *Aspergillus niger* on a Nitrate Medium.*

	Molybdenum content (mμg)			
μg W added	1.0	2.5	3.5	5.0
0	437	572	683	712
.1	410	510	574	675
.5	228	432	454	562

* mg dry wt of mycelium after 6 days.

required. Tungstate has been shown to be a dietary antagonist of molybdate in animal nutrition(5), and this report presents evidence that tungstate also functions as a competitive inhibitor of molybdate in *Aspergillus niger*.‡

Methods. The microbiological assay for molybdenum, as described by Nicholas(6), was the procedure used in these studies. Each 500 ml pyrex assay flask contained 50 ml of culture solution, freed of Mo by coprecipitation on CuS, plus 10 ml samples of sodium molybdate and/or other salts in appropriate concentrations. After inoculating with *A. niger* spores and incubating for 6 days at 25 ± 0.5°C, the mycelial felts were harvested, washed, dried at 80°C overnight and weighed. When the culture medium was adequate in all nutrients except Mo, and when nitrate was the sole nitrogen source, the dry weight of the mycelium was dependent upon the availability of Mo.

Results. Table I shows that the addition of 0.1 to 0.5 μg of W (as Na_2WO_4) to a culture medium containing 1 to 5 x 10^{-3} μg of Mo (as Na_2MoO_4) depressed the growth of *A. niger*. Two micrograms of tungsten in a system containing 2.5 x 10^{-3} μg Mo per 60 ml prevented growth completely, and this inhibition was overcome entirely by increasing the Mo content to 1 μg (W:Mo ratio of 1:1). A W:Mo ratio of unity maintained normal growth at all levels tested, up to the highest W content of 10 μg per 60 ml. Molybdate concentrations in the nitrate medium were varied from 1.74 x 10^{-10} M to 8.69 x 10^{-10} M (1 to 5 mμg Mo per 60 ml), and the typical Lineweaver-Burk(7) plots of competitive

‡ The *Aspergillus niger* (Mulder strain) used in these studies was generously supplied by Dr. D. J. D. Nicholas, Long Ashton Research Station, University of Bristol.

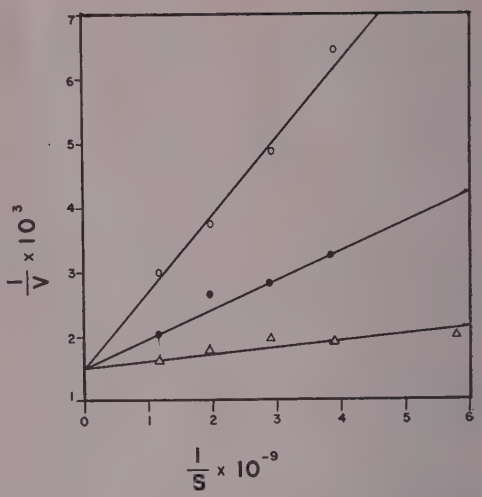

FIG. 1. Lineweaver-Burk plot showing competitive inhibition of molybdate by tungstate on growth of *A. niger* in a nitrate medium. V = mg dry wt of mycelium after 6 days. S = molar concentration of molybdate. △ = no tungstate. ● = 4.53 × 10^{-8} M tungstate. ○ = 9.06 × 10^{-8} M tungstate.

inhibition were obtained by adding tungstate at levels of 4.35 x 10^{-8} M and 9.06 x 10^{-8} M (0.5 and 1.0 μg W per 60 ml). Curves such as those shown in Fig. 1 were obtained and reproduced at various tungsten concentrations.

Molybdenum did not appear to be a necessary growth factor for this organism when ammonium ion was supplied as the nitrogen source (Table II). The basal medium was prepared by replacing the KNO_3 with an equivalent amount of NH_4Cl, and it was freed of trace amounts of molybdenum in the usual way(6). The ammonium medium not only supported better growth than the nitrate medium but the growth was unaffected by variations in Mo content. Growth on the

TABLE II. Effect of Mo and W on Growth of *Aspergillus niger* in Nitrate and Ammonium Media for 6 Days.

Metal content		mg dry mycelium	
mμg Mo	μg W	NO_3^- medium	NH_4^+ medium
0	0	140	993
0	2	—	1014
2.5	0	396	1010
2.5	2	29	1114
2.5	5	—	1111
1000	2	441	1088

nitrate medium containing 2.5 mμg of molybdenum was prevented by 2 μg of tungsten, but there was no decrease in growth with as much as 5 μg of W (the highest level tested) on the ammonium medium. Since growth was optimal on the ammonium medium and was unaffected by the Mo or W concentrations, the only apparent role for Mo in *A. niger* appears to be related to nitrate reduction.

Other ion relationships. Certain copper deficiency diseases in animals have been shown to be related to high dietary levels of molybdenum and inorganic sulfate(8,9). The converse effect of Cu or $SO_4^=$ on Mo deficiency was not observed with *A. niger*. On suboptimal levels of molybdenum (2.5×10^{-3} μg Mo) concentrations of copper or sulfate sulfur as high as 20 μg per 60 ml had no effect on growth. Manganous ion (40 μg/60 ml) also had no effect. Chromium (as K_2CrO_4) was studied in this system because of its association with Mo and W as a group VI_a element, but in molar Cr:Mo ratios as high as 15,000:1 chromium gave no growth inhibition. Bisulfite, tetraborate, chlorate, and ferricyanide were found to inhibit nitrate reduction by chicken liver xanthine dehydrogenase(10), a molybdoflavoprotein like nitrate reductase. However, when these substances were added to a nitrate growth medium containing 2.5×10^{-3} μg Mo in amounts which were equivalent on a molar basis to 2 μg W, no growth inhibition of *A. niger* resulted. Phosphotungstic acid was as effective a Mo antagonist as sodium tungstate, per equivalent of W.

Summary. When nitrate was the sole nitrogen source for *Aspergillus niger,* tungstate in a molar ratio of 20:1 with molybdenum readily produced growth inhibition, and a ratio of 400:1 prevented growth almost completely. The inhibition was entirely reversed by adding sufficient molybdate to give a W:Mo ratio of 1. The tungstate molybdate growth interaction yielded a competitive inhibition type of Lineweaver-Burk plot. The fungus displayed no requirement for molybdenum when grown on an otherwise adequate medium containing ammonium as the source of nitrogen.

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RO-1-7780, a Potent Antagonist of Alphaprodine.* (22528)

CHESTER W. WHITE, JR., ROBERT MEGIRIAN
AND PHILLIP S. MARCUS. (Introduced by E. L. Severinghaus.)
Anesthesia Research Laboratory, Boston City Hospital, and Department of Pharmacology, Boston University School of Medicine.

The narcotic antagonists have recently been reviewed(1). The hope that agents of this type would control undesirable side actions when given simultaneously with the narcotic has not been fully realized both because the time courses of action of the drugs used together have differed and because the antagonists have affected not only the undesirable actions of the opiates but the analgesia as well(2-12). Ro-7780‡ has received limited study previously(13-14); the purpose of the present work was to study its efficacy as an opiate antagonist and to determine its characteristics when used with alphaprodine (Nisentil) or morphine.

Methods. Ro-7780 was tested in conscious dogs and in dogs anesthetized with pentobarbital, 25 to 30 mg/kg. Male or female dogs weighing 8 to 12 kg were chosen. In addition a number of normal human volunteers and post-operative patients were given alphaprodine either alone or in combination with Ro-7780. All drug injections were made intravenously. Alphaprodine was administered in control studies on 6 conscious dogs; in later experiments in the same animals Ro-7780, 0.25 or 0.5 mg/kg, was given at the time of maximum apparent depression following administration of alphaprodine. Respiration, general activity and the effects of gross stimuli were observed. Two dogs also received 2 mg morphine per kg in similar studies. Experiments were next carried out to determine the effect of Ro-7780 in established alphaprodine depression in anesthetized dogs. The 9 animals used in this phase of the work were given pentobarbital and an endotracheal tube inserted. Expired gas volumes were measured using a wet test gas meter, arterial oxygen was determined according to Van Slyke and Neil using a manometric gas apparatus, and respiration and pulse were counted directly. Fifteen to 20′ after tracheal intubation in each animal alphaprodine (5-8 mg/kg) was injected. Artificial respiration was required for a period of 1 to 5 minutes in order to prevent death. On the resumption of spontaneous respiration, observations were made. Approximately 5 minutes later Ro-7780 (0.25 or 0.35 mg/kg) was injected and the measurements repeated immediately. In control experiments, which were carried out 7 to 10 days before or after the tests, Ro-7780 or no medication was given, and readings were made at the corresponding time intervals after anesthesia was induced. The same experimental design was used in 8 anesthetized dogs that received alphaprodine alone (4 or 5 mg/kg) in control experiments, while this dose of narcotic was given simultaneously with 1/75th or 1/100th the amount of Ro-7780 in test studies; arterial blood was drawn 5 to 8 minutes and 30 to 40 minutes following administration of the narcotic or of the narcotic-antagonist combination. In human subjects minute ventilation determined by an Emerson meter, respiratory and pulse rates, and blood pressure were recorded after alphaprodine alone (20-40 mg) in 4 cases and following combined administration of the same dose of alphaprodine with Ro-7780 at 1/75th or 1/100th the amount of narcotic in 6 cases. A period of 10 minutes with a variation in minute ventilation of less than 10% immediately prior to administration of the drugs was taken as the control and results were calculated as percent of this volume.

Results. In the 6 conscious dogs responsiveness was affected consistently while respiratory rate and pulse were variable and unpredictable. Alphaprodine by itself (2-10 mg/kg) always diminished the general ac-

* Supported by Hoffmann-LaRoche, Inc.

‡ This material was supplied by Hoffmann-LaRoche, Inc., under code number Ro-1-7780; and it is 1-3-hydroxy-N-propargyl morphinan tartrate.

TABLE I. Influence of Ro 7780 following Alphaprodine in 9 Dogs under Pentobarbital.

Pentobarbital alone*			Alphaprodine			Alphaprodine and Ro-7780		
Minute ventilation (L)	Respiratory rate	Arterial oxygen vol (%)	Minute ventilation (L)	Respiratory rate	Arterial oxygen vol (%)	Minute ventilation (L)	Respiratory rate	Arterial oxygen vol (%)
1.85	21	18.60	1.54	16	16.25	4.80	30	21.35
2.95	19	16.48	2.00	16	18.34	2.72	16	20.13
1.46	6	17.34	.94	7	14.40	4.70	23	20.44
1.49	16	16.97	.92	7	4.50	2.82	21	19.53
1.26	8	17.26	†			1.35	5	19.90
1.46	10	14.64	1.20	7	12.59	5.93	28	20.40
1.46	15	15.06	1.20	14	11.26	3.87	36	19.74
1.41	7	19.30	1.57	14	14.80	3.14	26	20.50
1.18	8	14.53	.93	6	10.15	2.27	17	16.56

Statistical comparisons by paired data:
A: Ventilation vol columns 1 cf. 4, $p < .02$; 1 cf. 7, $p < .01$; 4 cf. 7, $p < .01$.
B: Respiratory rate " 2 cf. 5, $p > .4$; 2 cf. 8, $p < .01$; 5 cf. 8, $p < .01$.
C: Arterial oxygen " 3 cf. 6, $p > .05$; 3 cf. 9, $p < .01$; 6 cf. 9, $p < .01$.

* Ro-7780 alone after pentobarbital had no effect on these measurements.
† Too severely depressed for measurements.

tivity and response to stimuli. Ro-7780 administered at the time of maximum depression after the narcotic produced a sharp increase in responsiveness in every animal. The animals that received morphine responded similarly to the antagonist.

In the 9 dogs under pentobarbital Ro-7780 (0.25 or 0.35 mg/kg) injected during a period of severe respiratory depression incident to the action of alphaprodine (5 to 8 mg/kg) produced a significant improvement in minute ventilation, respiratory rate and arterial oxygenation. The changes produced by alphaprodine alone were significant with respect to depression of minute ventilation and arterial oxygenation; but the spontaneous respiratory rate, after breathing was resumed, was not changed. Results from the individual animals are presented in Table I; paired data analysis was used for interpretation.† Ro-7780 had substantially no effect unless alphaprodine had previously been given. (On the basis of these experiments a somewhat lower dose of alphaprodine (4 or 5 mg/kg) was chosen as suitable in the subsequent animal work.)

Fig. 1 shows the mean results from studies of the combined administration of Ro-7780 and alphaprodine in dogs. Preliminary experiments had indicated that the antagonist should be given in a dose of about 1/75th to 1/100th the narcotic in order to prevent respiratory depression. The administration of alphaprodine by itself (4 or 5 mg/kg) produced a severe respiratory depression which lasted for about 12-15 minutes: 3 of the 8 animals, in fact, required artificial respiration for 1 to 7 minutes. When Ro-7780 was given contemporaneously with alphaprodine in the same animals, respiratory rate was significantly higher during this critical period (20 cf. 5), as were minute ventilation (1.45

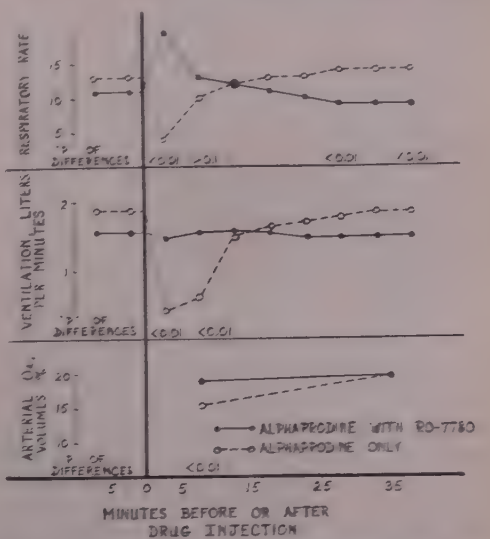

FIG. 1. Mean effects of alphaprodine alone and given simultaneously with Ro-7780 on 9 dogs (18 experiments).

† This and subsequent "p" values were calculated by Student's "t" test.

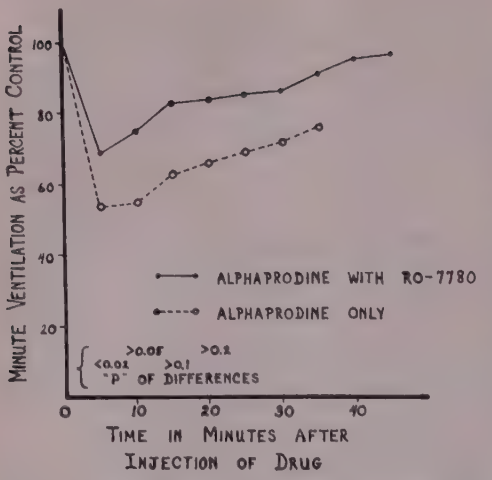

FIG. 2. Mean effects on minute ventilation of alphaprodine alone (4 subjects) and given simultaneously with Ro-7780 (16 subjects).

cf. 0.45 L/min.) and arterial oxygenation (19.8 cf. 15.2 vol. %). Statistical analysis of these data showed that Ro-7780 was effective in controlling the respiratory depression of alphaprodine. Although the figures for arterial oxygenation include the 3 dogs that were artificially ventilated after alphaprodine alone, nevertheless the mean oxygenation was significantly better in the group receiving the combined medication.

The mean effects of alphaprodine alone and combined with Ro-7780 in man are shown in Fig. 2. The use of the combined medication was associated with a significantly higher minute ventilation at the beginning of the test period. Although ventilation was improved, the patients who received antagonist were all either intermittently or constantly asleep during 30 or 40 minutes after the drug was given and had to be awakened for the observations.

Discussion. Since the original studies of Pohl on N-allylnorcodeine(15), several allyl narcotic derivatives have been investigated. These have included N-allylnormorphine (1-4), N-allylnorheroin (cited by Lasagna, 1), 1-allyl-4-phenyl-4-carbethoxypiperidine (11), and levallorphan(5-12). Ro-7780 is closely related to levallorphan, differing only by having a propargyl instead of an allyl substituent on the nitrogen.

A comparison of the potency of levallorphan and N-allylnormorphine, the two most widely studied antagonists, indicated that the former is at least twice as active as the latter. Costa and Bonnycastle(11) found both capable of preventing respiratory depression in rats given alphaprodine; as to relative potency, ratios of antagonist to alphaprodine of 1 to 20 for levallorphan and 1 to 7.5 for N-allylnormorphine were comparable. Swerdlow, Foldes and Siker(10) studied the combined effect of alphaprodine and levallorphan in humans and found ratios of levallorphan to alphaprodine of 1 to 50 or 1 to 100 adequate in correcting respiratory depression.

In our experiments on dogs a ratio of 1 to 75 or 1 to 100 of Ro-7780 to alphaprodine prevented respiratory depression. These ratios, however, did not completely restore normal respiratory exchange in man. They might have if larger doses of alphaprodine had been used, since antagonists appear more effective in more severely depressed subjects (9). Ro-7780 given either contemporaneously with or after alphaprodine produced an initial increase in ventilation rate and/or volume above control values in spite of the fact that Ro-7780 alone had no significant effect. Such action of opiate antagonists is in keeping with the reports of other workers(2,12).

It appears that Ro-7780 is a more potent opiate antagonist than N-allylnormorphine and at least as potent as levallorphan. Further studies would be necessary comparing the actions of the three agents under identical conditions in order to establish their relative potencies.

Conclusions. (1) 1-3-hydroxy-N-propargyl morphinan has been found a potent antagonist to alphaprodine or morphine in the dog and to alphaprodine in man. (2) Ro-7780 given contemporaneously with alphaprodine at 1/75th or 1/100th the dose of the latter appeared to counteract completely the respiratory depression produced in the dog by the narcotic alone. (3) At the same dose ratios of Ro-7780 to alphaprodine in man, the antagonist diminished the severity of the respiratory depression produced by alphaprodine,

but otherwise the narcotic effect was judged unaltered.

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Further Observations on Distribution of Radioactivity Following Parenteral Administration of Co^{60} Vitamin B_{12}.* (22529)

LEO M. MEYER, S. N. ARKUN AND M. JIMINEZ-CASADO.
New York University Service, Goldwater Memorial Hospital, N. Y. City.

It is now well established that following oral or parenteral administration of Co^{60} vit. B_{12} radioactivity may be detected over the liver, spleen and left kidney for periods up to 315 days(1-4). It has been further demonstrated that following the intramuscular injection of $Co^{60}Cl_2$ the pattern is entirely different, suggesting that uptake studies with the radiovitamin probably represent the B_{12} molecule in the organs surveyed(4).

The present report is concerned with further investigations on the distribution of Co^{60} vit. B_{12} under a variety of conditions: (1) following intravenous injection of the radiovitamin; (2) in association with large doses of "cold" B_{12} before, simultaneous with, or after intramuscular injection of the radiovitamin; and (3) administration of amethopterin prior to intramuscular injection of the radiovitamin.

Material and methods. The Co^{60} vit. B_{12} had a specific activity of 0.750 $\mu c/\mu g$. Each dose was diluted with sterile distilled water so that one ml contained one μg of radiovitamin. All administered doses were one ml in volume. Intramuscular injections were given in the right deltoid muscle, using a 1-inch 22 gauge needle. "Cold" vit. B_{12} was administered in the deltoid muscle of the opposite arm. A well-shielded sodium iodide crystal scintillation counter having an aperture of 2 cm was placed over (1) the site of injection (right deltoid muscle), (2) precordium, (3) lower right chest (liver), (4) lower left chest (spleen), (5) left lumbar region (left kidney), (6) lower end of sacrum, (7) lateral aspect of thigh. Radioactivity was recorded for 5-minute periods over each site within ½, 1, 2, 3, 4, 8, 12 and 24 hours after injection. Thereafter, measurements were made at 2, 3, 5 and 7-day intervals up to 168 days. Background counts were subtracted from observed values. No correction was made for Co^{60} decay. All patients were inmates of a chronic disease hospital and received essentially the same diets. Their ages varied from 43 to 80 years. The diagnoses included hypertensive heart disease (1), bronchial asth-

* Co^{60} vit. B_{12} and "cold" B_{12} were supplied by Merck and Co., Rahway, N. J. Amethopterin was supplied by Lederle Laboratories, American Cyanamid Co., Pearl River, N. Y.

TABLE I. Organ Distribution of Radioactivity following Parenteral Administration of Co^{60} Vit. B_{12}.

Treatment	No. of patients	Disappearance from site of inj. (days)	Days observation	Duration of plateau (days) Liver	Spleen	Kidney
A. Co^{60} B_{12}, intrav.	4	—	161–168	24–111	4–106	1–34
B. Co^{60} B_{12}, I.M. & cold B_{12} inj. for 7 days previously	2	3	120–127	104–113	120–127	6– 7
C. *Idem* & cold B_{12}, same time	4	½–3	53– 84	—	—	—
D. " " after 1 hr	2	1	13– 27	13– 27	6– 12 hr	3–12 hr
E. " " " 2 "	2	3–4	48– 70	7– 68	5– 12 "	5– 6 "
F. " " " 24 "	3	3–5	106	1– 91	3– 54	2 hr–20 days
G. " " " 7 days	4	—	86–101	86–101	64–101	1–87

ma (2), reticulum cell sarcoma (1), chronic lymphocytic leukemia (1), pulmonary fibrosis (1), rheumatoid arthritis (4), arteriosclerosis (8), gout (2), cirrhosis of liver (1), luetic heart disease (1), unilateral exophthalmus (1), and familial telangiectasia (1).

Results. The results are tabulated in Table I. Since no significant levels of radioactivity were noted over the precordium, sacrum and thighs, these sites were excluded from the table. The first column under each organ records the time after injection of the radiovitamin when maximal number of counts was noted.

(1) *Intravenous Co^{60} vit. B_{12}.* Four subjects received 1 μg of Co^{60} vit. B_{12} intravenously (Table I-A). Maximal activity appeared over the liver, left kidney and spleen in about 30 minutes, in contradistinction to hours and days respectively, noted following muscular injection(4). In all other respects the plateau and duration of activity in all 3 organs were similar to those noted following intramuscular administration.

(2) *Intramuscular Co^{60} vit. B_{12} associated with "cold" B_{12}.* (a) Two patients received 30 μg of "cold" vit. B_{12} for 7 days. This was followed by the intramuscular injection of 1 μg of Co^{60} vit. B_{12} on the eighth day (Table I-B). The data show that the pattern of distribution of radioactivity over liver, spleen and kidney was essentially the same as that previously reported from this laboratory when no "cold" B_{12} was administered. (b) Four subjects received 1 μg of Co^{60} vit. B_{12} intramuscularly in the right deltoid muscle. Immediately thereafter 1 mg of "cold" B_{12} was injected into the left deltoid muscle (Table I-C). The pattern of distribution of radioactivity was entirely different from those previously observed(4). Radioactivity disappeared from the site of injection in 12 to 72 hours. It reached its maximum over the liver in 2 to 3 hours. This level was of a low order, and only slightly above background counts. This low level of activity over the liver persisted for the duration of the study in each subject (up to 84 days). Radioactivity reached a maximum over the spleen in a short time (2 to 3 hours) but was again only slightly above background counts. Except for one case all detectable activity was gone in 4 days and no significant plateau was attained. Similar observations were made over the left kidney; *viz.*, maximal appearance in 2 to 3 hours, no plateau, and rapid disappearance over the left kidney in 3 subjects (6 hours to 2 days) and prolonged activity of 55 days in one case. (c). Two subjects received 1 μg of Co^{60} vit. B_{12} in the right deltoid muscle. This was followed 1 hour later by the intramuscular injection of 1 mg of "cold" B_{12} in the left deltoid muscle (Table I-D). The observed results were similar to those noted in experiment 2(b). Disappearance of radioactivity from the right deltoid muscle was delayed for 24 hours. The height of maximal activity over liver, spleen and left kidney never rose above the 1 hour post-injection level of the radiovitamin. The plateau was lower than that observed in previously described experiments(4). The plateau over the liver was maintained in both subjects for the duration of the experiment. Over the spleen and left kidney the plateaus were all short (maximum 12 hours), but ac-

tivity was detectable during the periods of observations. (d). Two patients received 1 μg of Co^{60} vit. B_{12} in the right deltoid muscle followed by 1 mg of "cold" B_{12} in the left deltoid muscle 2 hours later (Table I-E). The results in this study resemble those observed in experiment 2(c). Disappearance of the radiovitamin from the right deltoid muscle was delayed up to 96 hours after injection. Maximal activity over the liver was attained after 2 days, which was at a lower level than that generally observed in previous experiments(4), and only slightly higher than in experiment 2(c). Although radioactivity was detectable over the liver for the entire period of observation (48 and 72 days respectively) the duration of the plateau was extremely short in 1 case (7 days). (e). Three subjects received 1 μg of Co^{60} vit. B_{12} in the right deltoid muscle followed by 1 mg of "cold" B_{12} in the left deltoid muscle 24 hours later (Table I-F). Disappearance of radioactivity from the deltoid muscle was apparently not affected by the large dose of "cold" B_{12} as it fell within the range previously reported from this laboratory(4). However, maximal activity over the liver never rose above the 24 hour level and the plateaus in 2 of the 3 cases were only 1 and 3 days, respectively. In the third patient maximal activity was attained 1 day after injection of "cold" B_{12} in 1 patient, 2 hours later in another, and never rose above the preinjection level in the third. The plateau of radioactivity varied from 3 to 54 days. Detectable counts were present during the entire period of the experiment. Over the left kidney no further rise in counts was observed after administration of "cold" B_{12}. The plateau of radioactivity varied from 2 hours to 20 days. However, activity was present from 90 to 106 days. (f). Four subjects received 1 μg of Co^{60} vit. B_{12} in the right deltoid muscle. Seven days later 1 mg of "cold" B_{12} was injected into the right deltoid muscle. (Table I-G). The pattern of distribution and plateaus and duration of activity over liver and spleen were the same as those previously reported from this laboratory when no "cold" B_{12} was administered(4). Over the left kidney the plateau ranged from 1 to 86 days. Such extreme patterns were seen only in experiment 2(b), when 1 mg of "cold" B_{12} was given simultaneously with 1 μg of Co^{60} vit. B_{12}.

(3) *Prior administration of amethopterin.* Three subjects received 10 mg of amethopterin for 7 days, following which 1 μg of Co^{60} vit. B_{12} was injected into the right deltoid muscle. The patterns of disappearance of activity from the site of injection, uptake, plateau and duration over the liver, spleen and left kidney were similar to those previously reported from this laboratory when no folic acid antagonist was administered(4).

Discussion. From the foregoing results it appears that following intravenous injection of 1 μg of Co^{60} B_{12} the pattern of distribution of radioactivity over the liver, spleen and left kidney is similar to that observed after intramuscular injection. As might be anticipated, the time of initial and maximal appearance is earlier, probably because of direct introduction of the radiovitamin into the circulation.

The experiment in which the subjects received 30 μg of "cold" B_{12} for 1 week prior to intramuscular injection of Co^{60} B_{12} was set up with the purpose of "saturating" the organs with the vitamin, anticipating a slow or poor uptake of the radiovitamin. However, these results were not observed, suggesting that in the 24 hours between the last injection of "cold" B_{12} and the intramuscular injection of Co^{60} B_{12} enough vitamin was released from the liver, spleen and kidney to permit maximal deposition from 1 μg of Co^{60} B_{12} in the organs named. No corroboration for this hypothesis is, however, available. The studies with injection of Co^{60} B_{12} simultaneously with, or preceding, the administration of 1 mg of "cold" B_{12} by 1, 2 and 24 hours show what was expected; namely, a dilution effect of the large dose of non-radioactive vitamin on the absorption from site of injection and its deposition in liver, spleen and left kidney. Some of the inconsistencies in duration of plateau noted in the earlier report from this laboratory(4) were again observed, particularly in persons receiving the "cold" B_{12} 24 hours after the radiovitamin. The unanswered problem is the failure of the thousandfold

dose of "cold" B_{12} to dilute the radiovitamin so much so that none of it would be deposited or retained in the organs described. In the group of patients who received 1 mg of "cold" B_{12} 7 days after the injection of the radiovitamin there was no alteration in the pattern or plateau over the liver, spleen or kidney during the period of observation (up to 86 days). This would suggest that vit. B_{12}, once it reaches these organs, is not readily displaced by further administration of the vitamin.

The experiment in which amethopterin was administered for one week prior to Co^{60} B_{12} was investigated because of the intimate relationship of folic acid and vit. B_{12} in nucleoprotein synthesis. However, no alteration in the pattern of radioactivity distribution was observed. It is possible that a longer period of folic acid antagonist administration is necessary to affect vit. B_{12} deposition.

Conclusions. (1) Prior administration of 30 μg of "cold" B_{12} or 10 mg of amethopterin daily for 1 week, or injection of 1 mg of "cold" B_{12} 7 days after intramuscular injection of 1 μg of Co^{60} vit. B_{12}, does not alter rate of disappearance from site of injection or pattern of distribution of radioactivity over liver, spleen and left kidney. (2) Intravenous injection of 1 μg of Co^{60} B_{12} is followed by a more rapid appearance of radioactivity over the organs named. (3) Intramuscular injection of 1 mg of "cold" B_{12} simultaneous with 1, 2 or 24 hours after the administration of 1 μg of Co^{60} B_{12} reduces height of radioactivity over organs named but does not prevent its localization there.

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Role of Various Tissues in Metabolism of C^{14} Labeled Priscoline in the Rat.* (22530)

BERNARD CENTURY.† (Introduced by J. M. Coon.)
Department of Pharmacology, University of Chicago, Chicago, Ill.

Priscoline (tolazoline) hydrochloride is well known today, clinically as an effective peripheral vasodilating agent and pharmacologically as an adrenergic blocking agent. In a previous study, C^{14} labeled Priscoline administered to rats was rapidly excreted in the urine almost entirely unchanged(1). That a small amount of the drug is changed in the body was suggested by recovery of traces of C^{14} in the expired air and by the separation of small amounts of a radioactive metabolite in the urine. The method of analysis described in the present study permitted the detection of minute amounts of radioactive Priscoline and this metabolite in tissues as well as in urine, and made possible further investigation of the roles of various tissues in the turnover of Priscoline.

Methods and materials. The Priscoline used in this study had a specific activity of 1 μc/mg, and was labeled at the single benzyl carbon between the phenyl and imidazoline rings.‡ Sprague-Dawley albino rats weighing from 167 to 253 g were injected intraven-

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† National Science Foundation Postdoctoral Fellow. Present address: Biochemical Research Laboratory, Elgin State Hospital, Elgin, Ill.

‡ The C^{14} labeled Priscoline hydrochloride was very kindly supplied by Ciba Pharmaceutical Products, Summit, N. J.

ously with 7.95 mg/kg of radioactive Priscoline hydrochloride and were sacrificed after 1- and 2-hour intervals. Aliquots of urine and bladder washings and tissue homogenates were assayed for total C^{14} with internal gas flow Geiger counters. Tissues were analyzed for unchanged Priscoline and radioactive metabolites by the following procedure. To each 5 ml aliquot of tissue homogenate were added 25 ml of absolute methanol and 0.2 ml of concentrated ammonium sulfate. The supernatant was removed by centrifugation, and the precipitate was further extracted with 20 ml of absolute methanol. The protein precipitate contained no detectable C^{14}. The combined methanol extract was diluted to 50 ml, and a 15 ml aliquot was added to an equal amount of water in a separatory funnel containing about 2 mg of unlabeled Priscoline hydrochloride as a carrier. The 50% methanol solution was extracted once with 30 ml of carbon tetrachloride to remove fats and other substances soluble in organic solvents. No significant C^{14} was detected in this fraction. The 50% methanol fraction was made basic with ammonium hydroxide to a pH of over 11.1 and extracted 3 times with 30 ml portions of chloroform. The chloroform extract of each biological sample was evaporated to dryness, care being taken to avoid volatilization of the basic Priscoline. The radioactive residue was transferred with 4 small portions of ethyl acetate saturated with 0.1 M HCl onto a small Solka Floc or macerated cellulose column prepared with the same solvent system (2). An additional 14 ml of this solvent was used to elute a small radioactive fraction, while the unchanged Priscoline which remained on the column was eluted with 10 ml of a mixture of 55 parts of 95% ethanol, 45 parts of chloroform, and 1 part of acetic acid. Aliquots of the 50% methanol or aqueous fraction, the unchanged Priscoline, and the small chloroform extracted fraction were assayed for C^{14}. The results are expressed as millimicrocuries of C^{14}, which are equivalent to micrograms of administered Priscoline hydrochloride. Samples of urine were similarly analyzed, except that the precipitation of proteins was omitted. Control analyses were performed on samples of various tissue homogenates and urine to which known amounts of radioactive Priscoline were added.

Slices of liver, kidney, lung, spleen, and heart were freshly obtained from untreated normal rats and incubated 2 hours at 38°C in Warburg vessels containing 3 ml of Krebs-Ringer phosphate solution (pH 7.4 with C^{14} labeled Priscoline at a concentration of 9.44 μg/ml. Rat diaphragm and 0.2 ml samples of plasma were similarly incubated. The center wells of the flasks contained 0.2 ml of 10% KOH. The gas phase was oxygen, and O_2 consumption was measured during the first hour. Following incubation, the slices were removed, quickly blotted, homogenized in water, and aliquots were dried on copper planchettes for the determination of dry weights and C^{14} contents of the tissues. Wet weights were estimated by conversion factors separately determined for each tissue. A 2 ml sample of buffer from each flask was analyzed for total C^{14}, unchanged Priscoline, and radioactive metabolites. Similar analyses were performed on control buffers with radioactive Priscoline which were incubated without tissue samples and on unincubated solutions containing 0.2 ml of plasma. Liver slices were also incubated for 1 hour with radioactive Priscoline in Krebs-Ringer bicarbonate buffer under anaerobic conditions. The gas phase was 5% CO_2 in nitrogen. Liberation of CO_2 was measured manometrically. Slices and buffers were analyzed as above. Liver, kidney, heart, and spleen homogenates were also incubated in media containing radioactive Priscoline. Final concentrations of constituents in each flask were 0.1 M KCl, 0.02 M sodium phosphate (pH 7.4), 1.33×10^{-4} M cytochrome c, 0.001 M Na-ATP (neutralized), 0.0033 M $MgCl_2$, 0.01 M Na-pyruvate, and 0.004 M Na-fumarate. Eighty mg of wet tissue were added as 10% homogenates in 0.154 M KCl, and enough water was added to bring the volume to 3 ml. The gas phase was oxygen and the incubation time was 1 hour. The center well contained 0.2 ml of 10% KOH. Incubated homogenates were analyzed for total C^{14}, unchanged Priscoline,

TABLE I. Recovery of Unchanged Priscoline and Radioactive Metabolite from Tissues and Urine from 5 Rats Sacrificed 1 Hour after Injection and from 3 Rats Sacrificed after 2 Hours.

	Hr	C^{14}, mμc/g tissue	50% methanol soluble fraction		Unchanged Priscoline	
			mμc/g	% of total conc.	mμc/g	% of total conc.
Liver	1	16.6	2.34	14.1	12.4	75
	2	10.6	2.82	26.6	6.7	62
Kidney	1	22.9	.95	4.2	18.0	79
	2	13.1	.80	6.1	11.0	84
Lung	1	8.3	tr*	2.0	7.4	89
	2	4.29	n*	—	3.73	87
Spleen	1	6.04	tr	2.0	5.23	87
Heart	1	4.25	n	—	3.62	85
Skeletal muscle	1	4.66	n	—	4.36	94
	2	2.80	.14	5.0	2.62	94
Skin	1	2.78	n	—	2.51	90
Small intestine	1	8.05	.36	4.5	6.53	81
	2	5.10	.42	8.2	4.02	79
Plasma	1	2.28	.26	11.5	1.74	76
	2	1.25	.18	14.4	.92	74
		% of total dose	% of total dose	% of total recovery	% of total dose	% of total recovery
Small intestine content	1	3.31	.25	7.5	2.70	82
	2	1.95	.40	20.5	1.38	71
Urine	1	36.4	.55		31.8	
	2	59.7	1.55		55.4	

* tr = trace; n = negative.

and radioactive metabolites.

Results. Recovery of unchanged Priscoline and metabolites from tissues of treated animals. The total C^{14} content of tissues decreased rapidly and was excreted in the urine, as was previously reported(1), and most of the C^{14} in tissues and urine was recovered as unchanged Priscoline (Table I). The highest concentrations of C^{14} and unchanged Priscoline were found in liver and kidney tissues. Of significance is the increasing proportion of the 50% methanol or aqueous radioactive fraction found in liver and small intestinal contents. About 27% of the liver C^{14} was found in this fraction after administration of labeled Priscoline. A small but significantly increasing amount of the aqueous fraction was again found in the urine(1). In control studies, no detectable C^{14} was found in the 50% methanol fraction when labeled Priscoline was added to tissue homogenates and analyzed, so that all recoveries of C^{14} in this fraction in experimental tissues were considered significant. Small amounts of C^{14} in all samples analyzed were recovered as the chloroform extracted fraction which was separated chromatographically from Priscoline. In control analyses, about 1% of the added C^{14} was recovered as this fraction, and represented either contamination, impurities, or a small error inherent in the method. *Effects of rat tissues in vitro on radioactive Priscoline.* The ratios of concentrations of C^{14} in the tissue slices to those of the respective media were taken as a measure of the ability of the tissues to concentrate the radioactive materials from solutions containing labeled Priscoline. Liver and kidney slices took up averages of 20.2 and 26.8 mμc/g, respectively, which were 2.6 and 3.4 times the corresponding C^{14} concentrations in the baths. These were significantly higher ($p<0.05$) than similar tissue to bath C^{14} ratios for lung, spleen, muscle, and heart, with averages from

1.4 to 1.7. The media obtained following incubation with various tissues were analyzed for unchanged Priscoline and the two other radioactive fractions. From 90 to 95% of the C^{14} was recovered in the fractions as compared with the total C^{14} determinations. This was almost entirely unchanged Priscoline. The only finding significantly different from control analyses was the recovery of 1.4% of the total C^{14} in the bath as the 50% methanol fraction when liver slices were incubated aerobically with radioactive Priscoline. There was only a slight indication that kidney slices might be able to form this fraction. Small percentages of C^{14} were recovered as the chloroform extracted product, but no differences were seen from control analyses. There appeared to be no correlation between the Qo_2 of any tissue and its effect on Priscoline.

Under anaerobic conditions, liver slices incubated with radioactive Priscoline did not form the 50% methanol soluble fraction. However, the average tissue slice to buffer C^{14} ratio was about 3.5, indicating that the tissue retained its ability to concentrate the radioactive material. The average Qco_2 was about 2.5.

Liver, kidney, spleen, and heart homogenates were incubated with radioactive Priscoline for 1 hour. Four or more samples were run for each tissue. Analyses of the media indicated that no radioactive metabolic products were formed during this time. The average Qo_2 values for the homogenates were 19.4 for liver, 27.4 for heart, 53.3 for kidney, and 4.5 for spleen, based on a standard of 10 mg of wet weight tissue for 20 minutes.

Discussion. The results described here further indicate that the kidneys are the most important organs for the removal of Priscoline from the body(1,3). Analyses of various tissues and urine have shown that almost all of the administered drug is widely distributed in the body and excreted unchanged in the urine. A very small amount of the drug, however, appears to be metabolized by the liver to a product which is not extracted from alkaline aqueous solutions with chloroform, suggesting that the basic or alkaloid-like characteristics of Priscoline have been chemically lost. The appearance of increasing percentages of this 50% methanol soluble radioactive fraction in the liver ($p<0.05$) and small intestinal contents ($p<0.001$) and increasing amounts in the urine ($p<0.001$) suggest that it is slowly formed in the liver, excreted in the bile, reabsorbed by the intestine, and finally excreted in urine with the rest of the Priscoline. That the liver is capable of metabolizing Priscoline at a slow rate is further suggested by the recovery of a small amount of the same radioactive metabolic fraction following incubation of Priscoline with liver slices under aerobic conditions. Both intact liver tissue and oxygen appear to be necessary for the formation of this fraction. An oxidative degradation of the imidazoline ring is suggested by the loss of the chemically basic character of the molecule, and by the appearance of very small amounts of C^{14} in expired air(1). The positive inotropic action of Priscoline on the guinea pig heart and the inhibition of respiration of rat kidney slices have been reported as bioassay methods for determining concentrations of Priscoline in incubation solutions(4). Liver slices were reported to detoxify solutions of Priscoline at initial concentrations of 10 mg/ml. On the basis of experiments presented here, it appears that the decrease in biological potency observed by these investigators was almost entirely due to uptake of Priscoline by the liver slices, rather than to any metabolic action of the tissues.

Summary. Further studies on *in vivo* and *in vitro* metabolism of Priscoline are reported. Analyses of tissues, small intestinal contents, plasma, and urine of rats given radioactive Priscoline hydrochloride intravenously showed that almost all of the C^{14} was recovered as unchanged Priscoline both 1 and 2 hours after injection. Liver, small intestinal contents, and urine contained small but significantly increasing percentages of a 50% methanol soluble radioactive metabolite. About 27% of the C^{14} in liver was found as this fraction after 2 hours. When rat tissue slices were incubated with radioactive Priscoline under oxygen, liver and kidney took up the highest concentrations of C^{14}. Liver slices metabo-

lized small amounts of the drug to the 50% methanol soluble fraction under aerobic conditions, but not under anaerobic conditions. There appeared to be no correlation between Q_{O_2} values of tissues and their particular role in the disposal of Priscoline.

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Chemical and Hematological Studies on Blood of Bovine Dwarfs.*† (22531)

C. E. CORNELIUS, W. S. TYLER, AND P. W. GREGORY.‡
School of Veterinary Medicine and Department of Animal Husbandry, University of California, Davis.

Dwarfism in cattle has been reported since 1860 among many breeds of cattle in both *Bos taurus* and *Bos indicus*(1). Craft and Orr described an undersized Hereford steer with dwarf-like appearance and suggested this condition might be due to an underdeveloped thyroid and pituitary gland(2). A dwarf form morphologically similar to the preceding one has been studied more recently by various workers including Johnson, Harshfield and McCone(3), and Gregory and coworkers(4,5). This type of dwarf has a short broad head, an excessively bulging forehead, and marked prognathia. In addition to its small stature, it has a distended abdomen, a strong predisposition to bloat and breathes heavily. A premature fusion of the spheno-occipital synchondrosis in this dwarf type has been observed by Julian(6).

Due to the abnormal osteology in the dwarfs studied and their similarity phenotypically to mammalian cretins, this study of 38 "short-headed" dwarfs was initiated to understand more fully the pathological physiology present. Serum electrolytes and protein levels were run to aid in interpretation of osteological abnormalities. Cholesterol and protein-bound iodine determinations were used to assay thyroid function. Due to the lack of information on the hematology of the dwarf, blood studies were also included. The dwarfs were of both sexes and of both Hereford and Angus breeding.

Materials and methods. Samples were collected by jugular puncture prior to sacrificing for anatomical studies. All whole blood was collected in Heller and Paul's anticoagulant mixture for hematological studies. Standard methods were used for all blood counting procedures. Packed cell volumes were performed using Wintrobe's hematocrit tube. A Leitz Rouy Photometer was employed for hemoglobin determinations, using a wave length of 550 mμ, standardized by Wong's method for iron. Two hundred white blood cells were counted in all differential counts. Serum protein fractionations were carried out by the method of Wolfson, Cohn, Calvary and Ichiba(7) taking care to shake gently the span ether in the albumin determination. Saifer and Zymaris(8) have shown this prevents denaturation and approximates electrophoretic separation closely. Serum calcium was determined by the method of Clark and

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Collip(9), serum magnesium as by Levinson and MacFate(10), and inorganic phosphorus as recommended by Fiske and Subbarrow (11). A Coleman Junior Spectrophotometer model 6A was used for all spectrophotometric analyses. Cerebrospinal fluid was drawn by lumbar puncture and protein levels analyzed as by Johnson and Gibson(12).

Standard statistical methods were used and the mean ($\overline{X}$) and standard deviation (σ) are reported in all cases where values do not deviate from the normal. Deviations from normal in the blood differential counts also include standard error (ϵ), range (R), and random probability.

Results. The following data represent results from determinations on 38 Hereford and Angus dwarfs ranging in age from 6 days to 14 months of age: ($\overline{X} \pm \sigma$) cholesterol (156 ± 50.4 mg%), calcium (10.4 ± 1.2 mg %), phosphorus (7.6 ± 1.9 mg %), magnesium (3.25 ± 1.1 mg %), total protein (6.03 ± 0.9 g %), albumin (2.7 ± .67 g %), α globulin (0.74 ± 0.26 g %), β globulin (1.49 ± 0.55 g %) and γ globulin (1.05 ± 0.26 g %). Four serum protein-bound iodine determinations were 3.2, 3.7, 4.5 and 2.8 μg %.§ Five cerebrospinal fluid protein levels were 26.2, 22.8, 15.5, 28.0 and 55.0 mg%.

Hematological values are as follows: ($\overline{X} \pm \sigma$) packed cell volume (38.1 ± 0.5%), hemoglobin (11.7 ± 1.4 g %), W.B.C. (8.8 ±2.9 x 10^3/cmm), and R.B.C. (9.8 ± 1.6 x 10^6/cmm). In differential counts, lymphocytes are (%): $\overline{X}$ 55.6, σ ± 12.7, ϵ ± 1, and R 31-77. Neutrophils are (%): X 33.3, σ ± 12.5, ϵ ± 2.85 and R 10.5-58. Eosinophils are (%): $\overline{X}$ 2.1, σ ± 2.9 and monocytes (%): $\overline{X}$ 7.7, σ ± 3.5.

Discussion. Calcium, magnesium and phosphorus levels in the bovine dwarfs agree well with accepted normal values given by McSherry and Gryner(13) as well as those found in our own laboratory. Total protein and protein fractionations also appear to be normal. Both cholesterol and protein-bound iodine values are in the normal range and indicate that the "short-headed" bovine dwarf is not a primary thyroid cretin. Elevation in blood cholesterol levels could be expected if primary thyroid deficiency were present. Carroll *et al.* reported that a thyrotropic hormone deficiency was present in dwarf beef cattle(14). It is well known that hypopituitarism in rats is not associated with any marked change in plasma cholesterol levels (15,16). There still appears to be no obvious explanation for the fact that hypophysectomy does not cause an increased plasma cholesterol level as hypothyroidism in pituitary deficiency is very conspicuous. Protein-bound iodine determinations, therefore, appear to be better indices of thyroid function.

All hematological data agree with normal values of beef cattle under 14 months of age, as compiled by Schalm in our laboratory, except for the differential blood cell counts(17). Lymphocytes in normal beef cattle at this age are (%): $\overline{X}$ 68.0, σ ± 8.5, ϵ ± 1.6 and R 12-80. Neutrophils are (%): $\overline{X}$ 23.7, σ ± 7.7, ϵ ± 1.51, and R 12-38. The differences between normal and dwarf neutrophils and lymphocytes are highly significant with a random probability of less than 0.1% in both cases. Since total white counts do not vary from the normal, differences in the differential counts are not relative changes.

Summary. 1. Serum proteins, calcium, magnesium, and phosphorus of "short-headed" bovine dwarfs are all within normal limits. 2. Serum cholesterol and protein-bound iodine levels are within the normal range and indicate that the "short-headed" bovine dwarf is not a primary thyroid cretin. 3. All hematological values appear normal except for the differential count. Deviations from the normal are discussed.

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Factors Affecting Extraction and Recovery of Follicle Stimulating Hormone From Sheep Pituitary Glands.* (22532)

JOHN LEONORA, W. H. MCSHAN AND ROLAND K. MEYER.
Department of Zoology, University of Wisconsin, Madison.

A method was reported previously for extracting gonadotrophin from dry sheep pituitary tissue with saturated solutions of NaCl and KCl at 70°C(1). Further work resulted in a short simple procedure for obtaining follicle stimulating hormone by extracting pituitary tissue with saturated NaCl solution, removal of a protein fraction from the extract at pH 2.5, dialysis to remove the salt from the soluble fraction, and recovery of the activity quantitatively by use of an anion exchange resin(2). This procedure provides a method for obtaining relatively large amounts of follicle stimulating hormone (FSH) essentially free of luteinizing hormone (LH). The basic steps in this procedure were studied to determine the optimum conditions for increasing the yield and/or biological purity of the FSH preparation. The factors studied were concentration of salt and pH during extraction, and temperature and pH during fractionation. These factors were varied as indicated in the procedure, and the results obtained are reported in this paper.

Materials. Acetone dried whole sheep pituitary tissue was the starting material for these experiments. The NaCl solutions used for extraction were 80, 90 and 100% saturated. The saturated solution was prepared by shaking for one hour a given volume of distilled water with an excess of NaCl. The 80 and 90% saturated solutions were prepared by diluting the saturated solution with the proper volume of distilled water. The anion exchange resin XE-59 and the cation exchange resin XE-97 were obtained from the Rohm and Haas Co., Philadelphia, Pa.

Method of preparation. The amounts of pituitary powder extracted varied from 10 to 100 g but in most cases either 50 or 100 g were used. The dry pituitary tissue was mixed with the salt solutions in the ratio of 1 g of powder to 10 ml of the proper concentration of NaCl solution. The mixture was adjusted either to pH 4, 5, 5.5 or 6 with concentrated HCl or to pH 7, 8, 9 or 10 with NaOH. The mixture was allowed to stand for

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at least 20 minutes to reach equilibrium and a stable pH value. The mixture was maintained at 70°C for 1 hour by heating in a water bath during continuous stirring. The extract was recovered by centrifuging and the residue was extracted a second time in the same way. It was necessary to readjust the pH on the alkaline side for the second extraction. The 2 extracts were combined and allowed to cool to 30°C before adjusting to pH 2.5 by adding concentrated HCl dropwise while stirring. A few extracts were adjusted either to pH 3.0 or to pH 2.0. A heavy precipitate which formed at these pH values contained considerable follicle stimulating and luteinizing activities. This precipitate was utilized for other purposes. In some experiments the fractionations were also done at 50°, 60° and 70°C. The extraction was done as described above. After the first extract was recovered by centrifuging it was heated again to the desired temperature, adjusted immediately to pH 2.5 with HCl and centrifuged. The residue obtained after the first centrifugation was extracted again and the second extract was fractionated in the same way. The two fractions soluble at pH 2.5 were combined. The pH 2.5 soluble fractions

TABLE I. Effect of Follicle Stimulating Hormone Preparations Prepared at Different pH and Salt Concentrations on Ovaries of Normal Female Rats. (Fractionations were done at 30°C.)

				Concentration of NaCl solution used for extraction					
				% saturation					
				100		90		80	
				Assay of fractions—wt of glands (mg)					
Prep. No.	Extraction pH	Fractionation pH	Dose, g eq.	Protein, mg/g eq.	Ovaries*	Protein, mg/g eq.	Ovaries	Protein, mg/g eq.	Ovaries
A1	4	2.5	.5	8.6	14 ± .9†(9)‡				
			1.0		17 ± .2 (6)				
			2.0		14 (3)				
A2	5	2.5	.5	4.9	26.5± 1.3 (21)	4.6	34±4.1 (6)	5.7	40± 2.8(6)
			1.0		45.7± 1.8 (18)		53±6.0 (6)		117± 3.3(6)
			2.0		86 ±14.4 (5)				
A3	5.5	2.5	.5	4.8	40 ± 3.5 (6)				
			1.0		84 ±28.7 (6)				
A4	6	2.0	.5			3.6	74±8.0(13)		
		2.5	.5	5.5	38 ± 2.6 (20)	3.8	116±6 (15)§	4.6	102± 6.9(6)
			1.0		69 ± 5.6 (20)		147(3)		145±19 (6)§
			2.0		103 ± 9.0(11)§				
		3.0	.5			20.4	112±8 (24)§		
			1.0				116±9 (9) §		
A5	7	2.5	.5	4.3	60 ± 3.8 (32)	4.2	96(3)		
			.5			1.0	116(3)‖		
A6	8	2.5	.5	3.9	46 ± 3.2 (6)				
			1.0		120 ±10 (6)§				
A7	9	2.5	.5	7.0	65 ±14.3 (6)§				
			1.0		132 ±16.3 (6)§				
A8	10	2.5	.5		14 ± .9 (6)				
			1.0		15 ± 1.1 (6)				
A8 + FSH			.5		109 (3)				
			.5						
Idem			1.0		106 (3)				
			.5						
FSH			.5		118 (3)				
Control (uninjected)					17 ± 1.5 (8)				

* Ovaries contained only follicles unless otherwise designated.

† Stand. error of mean in this and subsequent tables.

‡ Numbers in parentheses in this and subsequent tables indicate No. of animals used for assaying preparations.

§ Ovaries contained 1-7 corpora lutea/ovary in ⅓ or less of animals in each group.

‖ This fraction was purified with anion exchange resin XE-59 and as indicated 96 mg ovaries were obtained before and 116 mg after purification.

TABLE II. Effect of Follicle Stimulating Hormone Preparations Prepared at Different pH and Salt Concentrations on Ovaries, Testes and Accessories of 29-Day-Old Hypophysectomized Female and Male Rats.

			Concentration of NaCl solution used for extraction									
			% saturation									
			100				90				80			
			Assay of fractions—wt of glands (mg)									
Prep. No.*	Ext'n pH	Dose, g eq.	Ovaries†	Testes	Ventral prostate	Sem. ves.	Ovaries†	Testes	Ventral prostate	Sem. ves.	Ovaries†	Testes	Ventral prostate	Sem. ves.
A2	5	5	113 (3)	728 (3)	7.7	4.1	206±41(5)	923±30(8)	12.9±1.4	6.3±.9	198 (2)	942 (3)	13.0	4.9
		10	118 (3)	811 (3)	8.7	4.6								
A3	5.5	5	118 (3)	875 (3)	11.5	4.1								
		10	159 (4)	867±63(6)	15.2±.8	6.7±.2								
A4	6.0	5	113 (3)	781 (3)	8.5	5.3	123 (4)	907 (3)	16.4	7.9	166 (2)	882 (2)	38.8	18.5
		10	210 (3)	988 (3)	13.2	5.2								
A5	7.0	5	145 (3)	746 (3)	13.5	6.3	204 (1)	1137 (3)	24	15.2				
		5					269‡(1)	1214 (3)	25.6	20.2				
Hypophysectomized rats—uninj. controls			6.4±.4(6)	191±10(11)	6.3±.4	5.2±.2								

* These preparations from Table I were fractionated at 30°C and pH 2.5. † These ovaries contained only follicles. ‡ This fraction was purified further with anion exchange resin XE-59.

TABLE III. Biological Effect in Normal and Hypophysectomized Rats of FSH Preparations Extracted at pH 6 and Fractionated at High Temperatures.

Method of preparation					Assay of fractions—wt of glands (mg)					
					Normal rats		Hypophysectomized rats			
Prep. No.	Extraction Salt conc., % sat.	Fractionation Temp., °C	pH	Protein, mg/g eq.	Dose, g eq.	Ovaries*	Dose, g eq.	Ovaries*	Testes	Ventral prostate	Sem. ves.
1	100	70	2.5	4.3	.5	20 (3)					
					1.0	29 (3)	10		844 (4)	13.4	5.1
2	100	60	2.5	4.2	.5	39 (3)	5	110 (2)	769 (3)	7.2	4.5
					1.0	58 (3)	10		985 (3)	12.1	6.1
3	90	60	2.0	4.9	.5	35 (3)					
					1.0	94 (3)	10		1086 (4)	16	6.6
4	90	60	2.5	4.5	.5	44 ± 1.8 (15)	5	209 ± 20 (7)	960 ± 40 (15)	14.3 ± .7	6.3 ± .3
					1.0	110 ± 9 (15)	10	205 (3)	948 ± 49 (8)	14.5 ± .7	6.8 ± .5
5	90	60	3.0	11.5	.5	59 (3)					
					1.0	118 (3)					
6	90	50	2.5	3.5	.5	42 (3)					
					1.0	82 (3)					
7	90	50	3.0	12.7	.5	72 (3)					
					1.0	72 (3)					
Uninjected control rats						17 ± 1.5 (8)		6.4 ± 4 (6)	191 ± 10 (11)	6.3 ± .4	5.2 ± .2

* These ovaries contained only follicles.

were dialyzed against distilled water and finally against 0.01% KCl solution to remove the NaCl. A light inactive precipitate formed during dialysis was removed by centrifugation. The fractions were dried by lyophilization. *Assay.* The fractions prepared by the above variations in the general procedure were assayed for gonadotrophic activity by using 21-day-old normal female rats of the Holtzman strain. The volumes of the extracts were adjusted with 0.9% saline solution so that each rat received the proper gram equivalent (g eq.) of dry pituitary tissue in 9 injections of 0.5 ml each. The first injection was made on the afternoon of the first day followed by injections on the morning and afternoon of each of the next 4 days. Autopsy was performed on the morning following the last injection. The ovaries were removed, dissected free of other tissues, weighed and examined by means of strong transmitted light for the presence of follicles and corpora lutea. These fractions were also assayed in male and female rats which were hypophysectomized when 27 days of age. Injections were begun when the rats were 29 days old, and were made on the morning and afternoon of each day for 10 days with autopsy on the morning after the last injection. The ovaries, testes, ventral prostate, seminal vesicles, thyroids and adrenals were removed, dissected free of other tissue, and weighed. The ovaries were examined macroscopically for the presence of follicles and corpora lutea.

Results. The gonadotrophic fractions obtained by extracting dry sheep pituitary tissue under different conditions of pH and salt concentration were assayed and the results from normal rats are summarized in Table I whereas those from hypophysectomized rats are given in Table II. These extracts were fractionated at 30°C and at pH 2.5 except for several extracts made at pH 6 with 90% saturated NaCl solution and fractionated at pH 2.0, 2.5 and 3.0. The data for saturated NaCl solution demonstrate that the amount of gonadotrophin extracted is dependent upon the pH over the range from pH 4 to 10. Inactivation occurred when the extracts were made at pH 4 (A1, Table I). The amount of FSH activity increased with increase in pH from 5 to 7. The ovaries produced by 0.5 and 1.0 g eq. of hormone contained only follicles (A2 to A5, Table I). The fractions from extracts made at pH 8 and 9 contained significant amounts of LH activity as shown by the fact that these ovaries contained corpora lutea (A6 and A7, Table I). Inactivation of FSH and LH occurred on extraction at pH 10 as shown by the lack of ovarian stimulation when the fraction was given alone and the lack of corpora lutea in the ovaries when it was given with FSH (A8, Table I).

Extractions were made with 90 and 80% saturated NaCl solutions at pH 5.0 and pH 6.0. Results obtained from the assay of these fractions and also those obtained with saturated salt solution show that when the pH was held constant the amount of hormone activity recovered increased as the salt concentration decreased (A2 and A4, Table I). The activity recovered at pH 6 was significantly greater than that recovered at pH 5 when fractions extracted with a given salt concentration are compared. When 90% NaCl extracts made at pH 6 were fractionated at pH 2.0, 2.5 and 3.0 there was an increase in activity with an increase in pH from 2.0 to 2.5 (A4, Table I). The ovarian response for the pH 2.0 fraction was only follicles whereas the response to the pH 2.5 and 3.0 fractions included a few corpora lutea in less than one-third of the ovaries. The protein contents of the pH 2.0 and 2.5 fractions were essentially the same (3.6 and 3.8 mg per g eq.) but the fraction obtained at pH 3.0 contained 20.4 mg per g eq.

The fractions (Table I) which appeared more promising in terms of being essentially free of LH were assayed in hypophysectomized male and female rats and the results are summarized in Table II. Doses 10 to 20 times greater than those given to normal female rats produced ovaries in the hypophysectomized rats that ranged in weight from 113 to 269 mg. These ovaries did not contain corpora lutea as determined by macroscopic examination. Since the ovary of the female rat is less sensitive to the action of LH than is the prostate to androgen pro-

duced by LH acting on the interstitial cells of the testes(4,5), the fractions of Table I were assayed in hypophysectomized males. When compared to those of uninjected control animals, the preparations made at pH 5 with saturated NaCl solution caused the least increase in ventral prostate weight of any of the fractions (Table II). When the pH was increased to 5.5, 6.0 and 7.0, the average prostate weights were about double the weight of those of the control animals. When the pH was held constant at 5.0 and the NaCl concentration was decreased from saturation to 90 and 80% saturation there was an increase in the weight of the prostate (A2, Table II). Similar results were obtained when the pH was held constant at 6.0 and the salt concentrations were decreased (A4, Table II). The fraction extracted with 80% saturated salt solution produced prostates that had an average weight of 38.8 mg which indicates that this fraction contained significant amounts of LH activity.

Another factor studied was the effect of fractionating at elevated temperatures. The rationale for these experiments is as follows. It was postulated that extracting with saturated salt solution at 70°C would dissociate weak protein linkages that might possibly result in releasing FSH from LH and other proteins(2). Furthermore, if this release of FSH by dissociation was reversible and was temperature sensitive, the dissociation might be greater at 70°C than at 30°C, the temperature at which the extracts were fractionated previously. On the basis of these assumptions it was thought that the yield of FSH might be increased by fractionating at 70° rather than 30°C and, also, that a cleaner separation from LH might be attained. The fractions were assayed in normal females and in hypophysectomized female and male rats. The results are given in Table III. The data from normal rats indicate that fractionation of the saturated NaCl extract at 70°C resulted in a great loss of FSH activity, whereas at 60°C considerable activity was recovered (1 and 2, Table III), but this was still less than that obtained at 30°C (A4, Table I). When 90% saturated NaCl extracts were fractionated at 60°C and at pH 2.0, 2.5 and 3.0 there was an increase in activity with an increase in the pH of fractionation (3 to 5, Table III). Fractions obtained at 50° and 60°C and at pH 2.5 contained essentially the same amount of activity (4 and 6, Table III). There was an increase in the activity when the fractionation was done at pH 3 and 50° and 60°C (5 and 7, Table III). Comparison of the ventral prostate response of the hypophysectomized male rats (Table III with results obtained from the same kind of rats (Table II) suggests that the fractions prepared at the high temperatures contained slightly less LH activity than those preparations made at 30°C. The ovaries from the hypophysectomized female rats contained only follicles.

Fraction 21A (Table IV) was obtained by extracting at pH 6 with 90% saturated NaCl solution and fractionating at 60°C and pH 2.5. It was further purified with anion exchange resin XE-59 according to the procedure reported previously by McShan *et al.* (2). The results substantiate further the effectiveness of the anion resin in eliminating a large amount of inert protein (81%) from fraction 21A without loss of FSH activity to give 21A(A-4). The latter fraction was fur-

TABLE IV. Biological Effect of a Preparation Fractionated at High Temperature and Purified with Ion Exchange Resins.

FSH preparation	Protein, mg/g	Normal rats		Hypophysectomized rats					
		Dose, g eq.	Ov. wt,* mg	Dose, g eq.	Testes, mg	Prostate, mg	Seminal vesicles, mg	Adrenals, mg	Thyroids, mg
21A	3.63	.5	43 (3)	5	936 (2)	10.6	5.7	13	4.3
				10	1156 (2)	17.6	7.9	13	4.3
21A (A-4)	.72	.5	46 (3)						
49F8	.118			10	952 (3)	11.6	5.1	6.7	3.6

* These ovaries contained only follicles.

ther purified by use of a column of cation resin XE-97(3) to give fraction FSH49F8 which contained only 0.118 mg of protein per g eq. of dry pituitary tissue. The results of the assay of the crude 21A and the purified FSH49F8 fractions in hypophysectomized male rats indicate that a significant amount of LH activity was removed by the purification steps as indicated by the decrease in the ventral postate weight from 17.1 mg for the crude to 11.6 mg for FSH49F8.

The weights of the adrenals and thyroids from the hypophysectomized rats used for assay of the various FSH fractions were not significantly different from the weights of these glands obtained from control uninjected rats. This indicates that the FSH preparations were free of ACTH and TSH at the high dosage levels used for the assay of the FSH.

Summary. (1) The effect of salt concentration and pH during extraction of sheep pituitary tissue, and temperature and pH of fractionation of these extracts on the preparation of FSH were studied. The most highly purified FSH from the biological standpoint was obtained by extracting at pH 5 with saturated NaCl solution and fractionating at pH 2.5 and 30°C. (2) The FSH preparations made by extracting with 90% saturated and saturated NaCl at pH 5, 5.5 and 6 and fractionating at 30°C and pH 2.5 contained little LH. (3) These preparations were free of ACTH and TSH at the levels tested. (4) The FSH can be concentrated and purified by use of anion and cation exchange resins. (5) Preparations made from 80% saturated NaCl extracts and from extracts made at pH 8 and 9 with saturated NaCl contained significant amounts of LH.

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Antagonists to Neuromuscular Block Produced by Sarin. (22533)

A. M. KUNKEL, J. H. WILLS AND J. S. MONIER.
Directorate of Medical Research, Army Chemical Center, Md.

The action of cholinesterase inhibitors on the neuromuscular apparatus has been explained by Burns and Paton(1) as originating by increased spread from the end-plate of the membrane depolarization normally elicited by ACh release. After complete cholinesterase inactivation, the end-plate region is thought to become sufficiently depolarized through the action of the ACh released by a single nerve discharge to be responsive no longer to indirect stimulation. The present work was undertaken to determine whether or not the block of neuromuscular transmission induced by comparatively large doses of Sarin can be overcome by chemical means, and whether any light on the mechanism of action of Sarin can be derived from a knowledge of chemicals capable of overcoming the neuromuscular block.

Methods. Sciatic nerves of cats lightly anesthetized with sodium pentobarbital were stimulated electrically with square wave stimuli at a frequency of 1 every 2 seconds, a pulse duration of 0.1 millisecond, and a supramaximal voltage (0.2 to 0.7 volt). Contraction of the gastrocnemius-soleus muscle group was recorded by a partially isometric lever. After control muscle responses had been obtained, the cat was given 200 μg/kg of Sarin through one antebrachial vein. Artificial respiration (interrupted positive pressure) was administered when required to maintain life. Five minutes later the treatment agent, if any, was administered by the

same route. If the treatment used was successful in restoring the muscle response to 90% of its original value within 30 minutes, a second dose of 300 μg/kg of Sarin was injected intravenously to determine whether or not the treatment had prophylactic as well as therapeutic value. Drug effects were measured by the times required for restoration of the twitch height to 50 and to 85% of its magnitude before Sarin administration. The significance of differences between mean times for the control groups and for the experimental groups was judged by Student's "t-test."

Results. The twitch height in animals given no treatment other than positive pres-

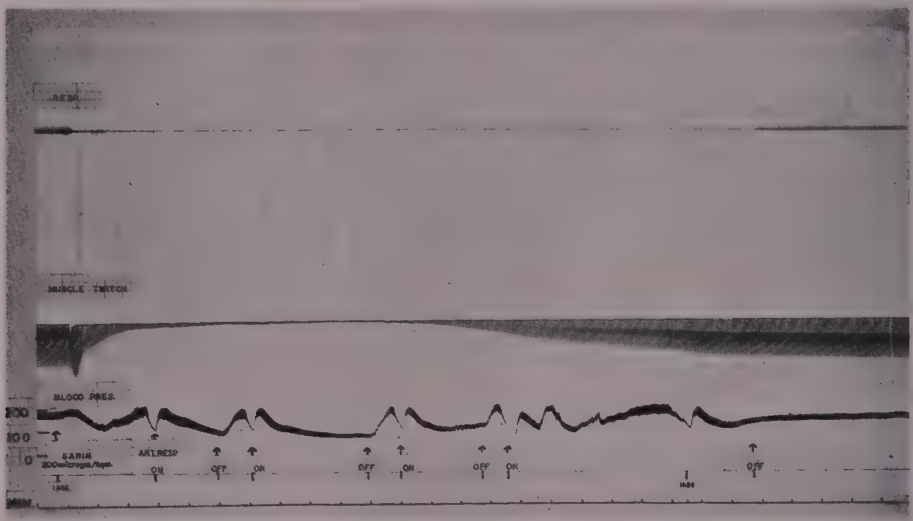

FIG. 1. Spontaneous return of twitch height to normal after Sarin, injected intravenously. Gastrocnemius-soleus muscle group of the cat, excited by just supramaximal electrical pulses. "Off" and "on" refer respectively to termination and to initiation of artificial respiration by a piston pump.

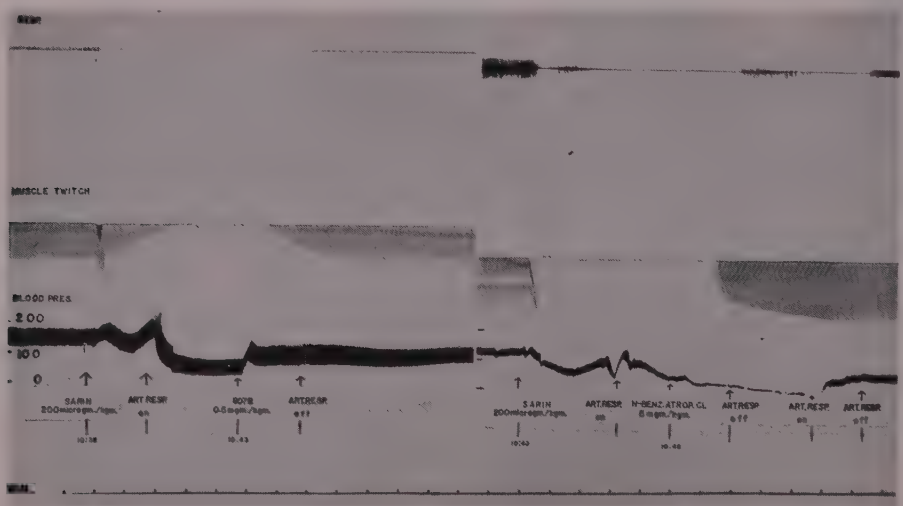

FIG. 2. Effect of WIN 8078 or of benzyl atropinium chloride on rate of return of twitch height to normal after Sarin. Conditions as for Fig. 1.

sure ventilation, as necessary, after Sarin administration returns slowly to normal (Fig. 1). Administration of a comparatively large dose of atropine (2 mg/kg, i. v.) 5 minutes after the poisoning with Sarin had no striking effect on the rate of return of the muscle twitch to normal magnitude. It was observed that quaternary derivatives of atropine, methyl atropinium nitrate and isopropyl atropinium bromide, had moderately potent, but temporary, effects in promoting return of the height of the muscle twitch toward normal. Two other quaternary derivatives of atropine, benzyl atropinium chloride and phenacyl atropinium bromide, were therefore made and tested. Both of these compounds produced a striking and lasting return of the twitch height toward normal (illustrated for benzyl atropinium chloride in Fig. 2). Other quaternary atropinemimetic compounds, like Lergigan methiodide and Dibutoline, also possessed this ability. None of these compounds conferred any significant protection against a second dose of Sarin given 30 minutes after the initial one.

Other compounds known to affect the neuromuscular junction (quaternary oxamide derivatives(2), d-tubocurarine, Flaxedil and decamethonium) were studied in the same way. D-tubocurarine and Flaxedil both increase very significantly the rate of return of the twitch height to 50% of its pre-poisoning value. The rate of return to 85% of the original contraction height was increased significantly by d-tubocurarine but not so significantly by Flaxedil. Decamethonium prolonged significantly the time for return to 50% of the original value and much less significantly the time for 85% recovery. Certain of the oxamides (WIN 8078, WIN 8626 and WIN 12306) had highly significant effects in shortening both times (Fig. 2), but a fourth one (WIN 8077) prolonged both times. Nicotine hydroxamic acid methiodide, a quaternary salt of one of the hydroxamic acids reported to be capable of reversing *in vitro* inhibition of cholinesterase by P-containing compounds(3), was found to enhance somewhat the recovery of twitch height when given intravenously in sufficiently large doses. Table I gives the mean values, for groups of 6 cats, of the times for recovery of 50 and 85% of the pre-poisoning twitch height and the corresponding "t" values for the differences between the control times and those for the animals given drugs. This table gives also information about the abilities of the various compounds to prevent the action on

TABLE I. Effectiveness of Various Compounds in Promoting Recovery of Twitch Response to Sciatic Nerve Stimulation following Injection of 200 μg/kg of Sarin. All treatment compounds injected intravenously 5 min. after Sarin.

Treatment compound	Dose, mg/kg	Minutes for 50% recovery	Minutes for 85% recovery	"t" drug *vs* control for 50% recovery	"t" drug *vs* control for 85% recovery
none	—	19.6	27.1		
atropine SO_4*	2.0	16.1	22.3	1.0	1.2
N-B A*	5.0	6.4	8.3	5.4	6.3
N-P A*	5.0	6.1	9.8	5.5	4.9
Dibutoline*	5.0	6.6	10.7	5.3	5.5
d-tubocurarine‡	.3	6.7	8.1	5.2	6.3
Flaxedil*	.25	7.1	16.9	5.0	2.1
decamethonium*	.01	25.4	30.0	2.4	1.0
WIN 8077†	.05	25.2	38.4	1.1	1.0
" 8078†	.5	6.5	9.0	5.4	6.0
" 8626†	.5	7.0	8.9	5.1	5.9
" 12306‡	1.0	6.4	8.0	5.4	6.4
NHAM*	35-70	14.0	20.7	1.8	2.1

N-B A = N-benzyl atropinium chloride. N-P A = N-phenacyl atropinium bromide. NHAM = nicotine hydroxamic acid methiodide.

* This compound protected none of 3 to 5 animals against a second dose of Sarin.
† " " " 1 or 2 out of 3 or 4 animals against a second dose of Sarin.
‡ " " " all of 4 animals against a second dose of Sarin.

twitch height of a second dose of Sarin. It is especially striking that the N-benzyl and N-phenacyl atropinium salts and Dibutoline had no prophylactic value against a second dose of Sarin although WIN 8078, WIN 8626 and WIN 12306, which were not strikingly more potent therapeutically, had definite, and in the last instance striking, ability to prevent the effect of a second dose of Sarin on the response to sciatic nerve stimulation.

Discussion. The foregoing findings may be applied to an examination of the idea of Cohen and Posthumus(4) that the P-containing anticholinesterases react primarily with an "esteratic" site of the receptor surface, thereby sensitizing an adjacent "anionic" site to acetylcholine. Compounds capable of being attracted to the "anionic" site by electrostatic charges, like those containing electropositive nitrogen atoms, are held to block the depressant effects of acetylcholine on the previously sensitized muscle by competitive occupation of the "anionic" site. This theory is fitted by all our findings except the one that decamethonium does not antagonize the Sarin-induced decrement in the response of the muscle to electrical excitation of the motor nerve. Cohen and Posthumus postulated that decamethonium reacts with the "esteratic" group, and this idea may be strengthened somewhat by the finding of Paton and Zaimis (5) that decamethonium is mildly inhibitory of cholinesterase. By reacting with the "esteratic" group rather than, or in addition to, the "anionic" group, decamethonium would enhance rather than antagonize the sensitization to acetylcholine induced by the P-containing anticholinesterases. It may be significant in the same regard that WIN 8077, which has been shown by Arnold *et al.*(2) and Lands *et al.*(6) to be both the most toxic compound and the most potent cholinesterase inhibitor of the 4 oxamides used here, is the only oxamide which prolonged the recovery times. The more weakly antiesteratic compounds may be assumed to be directed by their quaternized nitrogen atoms to the "anionic" site predominantly and WIN 8077 to be directed by its greater affinity for cholinesterase to the "esteratic" site predominantly. It is difficult for us to see how this mechanism could be in operation after administration of the large dose of Sarin used in our experiments, however; we have used 10-12 LD50's of Sarin intravenously, and find no demonstrable cholinesterase in the muscles. This leaves us, therefore, without explanation for the different behaviors of decamethonium and WIN 8077; one possibility would be that cholinesterase is not the sole point of attack of Sarin at the neuromuscular junction. Groblewski *et al.*(7) have found other evidence that the P-containing moieties of DFP and Sarin may have direct actions on the contractile mechanism in striated muscle without reference to cholinesterase.

Conclusions. 1. Certain compounds containing quaternary nitrogen atoms are able to overcome the decrease in twitch height, of the cat gastrocnemius-soleus muscle group excited by maximal electrical stimulation of the sciatic nerve at one shock per two seconds, induced by large doses of Sarin. 2. Compounds having significant anticholinesterase activity may enhance the Sarin-induced decrease in twitch height despite the abolition by Sarin of demonstrable cholinesterase activity in the muscle.

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Inhibition of Mammary Carcinoma 755 by Malonic Acid Derivatives.* (22534)

B. L. Freedlander, F. A. French and Arthur Furst.†
Research Laboratories, Mount Zion Hospital, San Francisco, Calif.

This paper presents an evaluation of a number of substituted malonic esters and amides which were tested as carcinostatic agents against mammary carcinoma 755 in C57 black mice. Malonic acid is a classical inhibitor of succinic dehydrogenase in the Krebs cycle. This inhibition may offer a basis for evaluating new types of malonic derivatives which are more selective in their action on cancer tisues than on normal tissues. Previous investigators have confined their efforts largely to malonic acid and amide on a single tumor. Boyland(1) found orally administered malonic acid, its ethyl ester and amide to be carcinostatic in dba mice with grafted mouse sarcoma. Gal(2,3) and coworkers reported several malononitriles including ethoxymethylene, p-nitrobenzilidene and 5-nitrofurilidene malononitrile moderately effective against the C3HS carcinoma. Other investigators(4) have found a number of malonic derivatives inactive on sarcoma 180. Petrakis(5) tested these compounds on human neoplasms with no therapeutic effect. The malonic esters and amides tested here were of the types $R\text{-}CH = CH(COOR)_2$ and $R\text{-}CH = CH(CONR_2)_2$. Five of these compounds, administered orally, showed some degree of activity (Tables I and II). These active compounds were also tested on other mouse tumors with negative results.

Methods. From 12 to 60 C57 black mice were weighed and then inoculated subcutaneously by trocar with mammary adenocarcinoma 755. These mice were divided into 2 groups; the first group was untreated and served as the control. For the second group, the drugs were mixed in the diet (Purina Laboratory Chow) at the maximum percentage the mice could tolerate with a slight weight loss. This value was determined by a preliminary 5-day toxicity test. Therapy was started the day of the tumor implant. At the end of 11 to 14 days the animals were weighed, the tumors were measured in 2 diameters, and the surface area was calculated. Experiments were repeated in a similar manner with sarcoma 180 in Webster-Swiss mice and with neuroblastoma C-1300 in strain A mice. The drugs were also evaluated on myeloid leukemia C1498 in C57 black mice, and were judged negative if the average longevity was prolonged less than 2 days compared to that of the controls.

The *results* are summarized in the Tables. The data for the inhibitory effect of 8-azaguanine (No. 17), a known inhibitor of mammary carcinoma 755 is given for comparison. Diethyl ethoxymethylenemalonate (No. 3) showed a definite carcinostatic effect; the treated mouse tumors were 16.5% as large as the untreated controls. This compound is relatively low in toxicity; the mice tolerated 1.2% of the drug in the diet. If therapy was deferred one day the positive effect was considerably decreased. Diethyl formylmalonate (No. 5) was also active, somewhat less than compound No. 3. The β-pyridylene derivative (No. 12) also showed some activity. The weight loss that accompanied the administration of these drugs was in general less than 10% of the body weight.

Of the malonamides, the amide of the most active malonic ester, namely ethoxymethylenemalonamide (No. 22) was only moderately active. The N, N, N′, N′-tetramethyl malonamide (No. 24) showed significant inhibitory effect on the tumor growth, whereas the tetramethyl amide of the ethoxymethylene compound (No. 26) had only slight activity. None of the substituted amino malonates had any effect on the transplanted tumor. These include the urea (No. 7), the thiourea (No.

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† Department of Pharmacology, Stanford Univ. School of Medicine.

8), the N-formyl (No. 10) and the N-acetyl (No. 11) derivatives. None of the compounds active against mammary carcinoma 755 showed any inhibitory effect against the other tumors tested (*i.e.* Sarcoma 180, Neuroblastoma 1300 and Myeloid Leukemia C1498).

Discussion. It is possible to draw some conclusions on structure-activity relationships from these results. The activity of diethyl ethoxymethylenemalonate (No. 3) may be related to the formation of the hydrolysis product diethyl formylmalonate (No. 5), which is also active. The inactivity of the formyl succinate ester (No. 16) implies that the malonate moiety is required. Unsaturation *per se* is not a requisite for activity.

TABLE I. Malonic Derivatives.

No.	$RC<^{COOC_2H_5}_{COOC_2H_5}$ Compound R	Dose % in diet	Surface area of tumor relative to controls, %	Ratio wt gain, treated/ controls	Ratio living, treated/ controls
1	$=H_2$	1.2	> 80	+0.5/+0.9	15/15
2	$=C_2H_5$	1.2	"	—1.1/+1.0	23/23
3	$=CH \cdot O\,C_2H_5$	1.2	16.5	—1.6/+2.1	32/33
4	$=CH_2$	.4	> 80	—0.4/+0.8	11/11
5	—CHO	1.2	30	—1.2/+1.6	13/13
6	$=CH\,CH_3$	.4	66	—0.2/+2.1	6/ 6
7	$=CH \cdot NH \cdot CO \cdot NH_2$	1.2	> 80	+0.4/+2.1	6/ 6
8	$=CH \cdot NH \cdot CS \cdot NH_2$	.9	"	—0.5/+2.4	6/ 6
9	$-NO_2$	.3	"	—0.5/+2.4	6/ 6
10	—NHCHO	1.5	"	—0.5/+2.4	6/ 6
11	$-NH \cdot COCH_3$	1.5	"	+0.5/+2.4	6/ 6
12	$=CH$ 	.4	40	—1.2/+1.4	20/21
13	$=CH$ 	1.2	62	—0.1/—1.0	7/ 7
	Related compounds				
14	$CH_3COC(=CH_2)—COOET$ α,Methylene acetoacetic ester	1.2	> 80	—1.7/+2.4	6/ 6
15	$ETOOC \cdot C(=CH_2)—CH_2COOET$ Ethyl itaconate	1.0	"	—3.7/+2.4	6/ 6
16	$ETOOC \cdot CH(CHO) \cdot CH_2COOET$ Diethyl formyl succinate	.6	"	—0.5/+2.5	6/ 6
17	8 Azaguanine	50 mg/kg i.p.	15	—2.3/+0.9	14/14

TABLE II. Malonamide Derivatives.

No.	$RC<^{CONR^1}_{CONR^2}$ Compound R	R^1	R^2	Dose % in diet	Surface area of tumor relative to controls, %	Ratio wt gain, treated/ controls	Ratio living, treated/ controls
21	H_2	H_2	H_2	.9	73	—0.1/+1.0	8/ 8
22	$=CH–OC_2H_5$	H_2	H_2	.8	48	—1.3/+0.4	16/16
23	H_2	CH_3	CH_3	.8	61	—0.8/+2.0	10/10
24	H_2	$[CH_3]_2$	$[CH_3]_2$	1.2	32	—0.1/+1.1	12/13
25	$=CH–OC_2H_5$	CH_3	CH_3	.9	>80	—0.7/+2.0	10/10
26	$=CH \cdot OC_2H_5$	$[CH_3]_2$	$[CH_3]_2$	.9	70	—0.6/+0.8	8/ 8

Neither the diethyl ethylidinemalonate (No. 2) nor the diethyl methylenemalonate (No. 4) is active; the unsaturated aceto acetic ester (No. 14) and itaconic ester (No. 15) are also inactive. The activity of the tetramethyl malonamide (No. 24) may be related to its greater lipid solubility. The ethoxymethylene grouping (No. 22) may impart polarity for a favorable partition coefficient.

The mode of action of these malonates might be explained by their interference with either of 2 biological pathways. Diethyl ethoxymethylenemalonate may be an inhibitor of succinic acid dehydrogenase, and consequently interfere with the tumor energy supply. Busch and Potter(6) found that succinate accumulated in tumor tissues of animals treated with sodium malonate. Another possible explanation for the activity may be considered relative to nucleic acid synthesis. It has been postulated, tentatively, that urea and oxaloacetic acid are involved in the synthesis of pyrimidines *via* orotic acid. It is conceivable that diethyl ethoxymethylenemalonate could either (a) block the condensation of urea and oxaloacetate, thus blocking orotic acid formation, or (b) condense with urea to give rise to an isomer of orotic acid, namely uracil-5-carboxylic acid. Either pathway for the utilization of diethyl ethoxymethylenemalonate might interfere with the build up of nucleic acids necessary for tumor growth.

Summary. Diethyl ethoxymethylenemalonate inhibited the growth of transplanted adenocarcinoma 755 in C57 black mice. The formyl and β-pyridylene malonic ethyl esters as well as two malonamides gave moderate inhibitory values. These compounds were inactive when tested against other transplanted tumors. Some possible modes of action are discussed.

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Preparation of Human Amnion Tissue Cultures.* (22535)

HASKELL J. WEINSTEIN, CAROLYN ALEXANDER, GEORGE M. YOSHIHARA, AND WILLIAM M. M. KIRBY.

Department of Medicine, University of Washington School of Medicine and King County Hospital System, Seattle.

Successful growth of human amnion in monolayer tissue culture was first reported by Zitcer *et al.*(1). A subsequent report from the same laboratory verified the usefulness of this tissue for culture of poliomyelitis virus (2). Because of the ready availability of placental tissue, attempts to culture human amnion were undertaken in this laboratory. Placentas from both vaginal and Caesarean section deliveries were used. These preliminary attempts were unsuccessful, however, and modifications of the method were undertaken, including a number suggested by Hok (3). The procedure here described evolved from these studies, and has proven to be reliable, simple, and economical. It requires little equipment that is not readily available in the average hospital laboratory. The tissue cultures are of uniformly good quality, and require a minimum of attention.

Method. 1) Human placentas from both Caesarean and vaginal deliveries have been collected by the obstetrical service of the King County Hospital System. Good growth

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is obtained from either source; however, only placentas from uncomplicated vaginal deliveries are routinely used. The perineum is prepared with antiseptic soap, followed by a voluminous saline rinse. The placenta is delivered directly from the vagina into a dry, sterile basin, avoiding contact with the perineum, rubber gloves and antiseptic solutions. After examination, the placenta is placed in a dry, covered sterile beaker. Placentas collected from deliveries occurring after midnite are stored at room temperature until brought to the laboratory in the morning. 2) The placenta is placed in a sterile pan, and the amnion is separated from the chorion by blunt dissection with forceps. The amnionic tissue is placed intact in a large petri dish with 50 ml of Hank's balanced salt solution containing 100 units penicillin and 100 μg streptomycin per ml, and adhering blood vessels, mucus, and other debris are removed with forceps. Occasionally it is necessary to scrape the amnion with a spatula to remove accumulated debris. The relatively clean amnion is then put in 3 or 4 consecutive washes of Hank's balanced salt solution for 5-10 minutes each, with occasional gentle stirring. Manipulation and squeezing of the tissue is kept at a minimum. 3) The specimen is placed in a 250 ml Ehrlenmeyer flask containing approximately 100 ml of .25% trypsin (Difco 1:250) in phosphate buffer solution(4). The preparation is left at room temperature for approximately 1 hour, during which the residual debris becomes suspended in the solution, which is then discarded. An equal amount of fresh trypsin is added, and the preparation allowed to remain at room temperature for an additional 4½ to 6 hours without agitation. The trypsin solution remains relatively clear until the digestion process nears completion. With release of cells from the amnion, the solution becomes cloudy, and the surface of the amnion appears soft and fuzzy. A quick shake of the flask aids in releasing cells from the tissue. The process is arbitrarily terminated when the fluid appears almost opaque. Subsequent trypsinization has yielded few additional cells. 4) The cell suspension is decanted through 1 layer of sterile gauze into graduated centrifuge tubes and spun for 10 minutes at 1000 rpm. The supernatant trypsin solution is discarded and the cell pack suspended in 15-20 ml of medium 199. The tubes are spun again at 800 rpm for 5 minutes and the supernatant discarded. Approximately .5 to 1.5 cc of packed cells have been obtained from the average amnion treated in this manner. 5) The cells are suspended in sufficient culture medium to make a 1:50 dilution. The medium employed has been 20% inactivated, filtered beef serum in medium 199† containing 200 units of penicillin, 200 μg of streptomycin, and 25 units of nystatin per ml. Cell counts average 5 million cells per ml in the 1:50 dilution. 6) Approximately 50 million cells, ordinarily about 10 ml of the above suspension, are further diluted with culture medium to 30-40 ml and pipetted into 1 liter Roux bottles, then incubated at 37°C. Within 48 hours, the living cells attach themselves to the glass wall, and the medium is replaced with an equal volume of fresh medium to eliminate floating debris. Subsequently, the medium is changed every 5 to 7 days, depending primarily on pH changes. In 7-14 days, a solid sheet of large, clear cells completely covers the glass wall, and the cells are ready for harvesting. 7) The medium is decanted, and 4 ml of 2% trypsin in Hank's solution, 16 ml of medium 199, and 4 ml of 2.8% $NaHCO_3$ are placed in the bottle. After incubation for 30-60 minutes, the cell sheet can be seen floating in the solution and the suspension is pipetted in graduated 40 ml centrifuge tubes and spun at 800 rpm for 2 min. The supernatant is decanted and 5 ml of medium are added to the cell pack, which usually measures .2 to .4 ml. The cells are dispersed with a pipette and more culture medium is added to make a 1:75-1:100 suspension of the cells. One ml of this suspension is inoculated into screw top tubes which are incubated on slant trays. For further propagation in large bottles, 20 ml of the suspension are used per bottle. These

† Medium 199 was generously provided by Dr. Ralph Houlihan, Cutter Laboratories, Berkeley.

secondary cultures are frequently of a better quality than the primary cultures, and grow considerably faster. In the tubes, there is often a solid sheet of cells within 72 hours, and the cultures are ready for virus inoculation or other studies. In bottles, growth may be adequate for further harvesting in 4-7 days. 8) Cultures can also be started directly in tubes, and for this purpose 200,000-300,000 cells in 1 ml of medium are inoculated into screw top tubes and incubated on slant trays. Culture medium is changed as described in section 6, but the 48-hr. change is not necessary. Growth is slightly faster in the tubes, being adequate in 7-10 days for further studies, but the quality of the cultures is usually better when the cells are first grown in bottles and then transplanted to tubes. 9) Tubes with satisfactory cell sheets, not needed for immediate studies, can be stored in an incubator at 28°C. The medium is changed every 15-20 days, and the cultures can be maintained without apparent damage for periods exceeding 6 weeks. 10) For virus studies, the culture medium is discarded, the cell sheet is washed with Hank's balanced salt solution, and a serum-free synthetic medium consisting of medium 199 with antibiotics is introduced. Control cultures can be maintained in this medium for 15 or more days before significant degeneration occurs.

Results. Good growth has been obtained with approximately 80% of the amnions treated in the above fashion. Attempts to perpetuate a strain of cells from a single amnion have not been successful. Good growth of cells was obtained through 4 subcultures on one occasion, but subsequent attempts to reproduce these results have failed.

Zitcer(1) described a mixed population of large and small cells in amnion tissue cultures, but in our material this has not been observed. The cells are all large, clear, quite uniform in shape and appearance with little or no granularity, and have a very obscure, faintly visible nucleus. Cytopathogenic changes result from infection with virus. The changes attributable to poliovirus consist primarily of the rounding up of cells, increased granularity, and darkening and prominence of the nucleus. Ragged holes of various sizes appear in the sheet of cells. These changes are similar to those observed in monkey kidney cells, except that relatively few of the amnion cells fall away from the glass. Infection with various strains of APC virus results in characteristic cytopathogenic changes in which the prominent feature is the development of long, hyalinized, spindle shaped cells. In addition, there is usually rounding up of cells in the periphery of the sheets, but few cells fall away from the glass. Cytopathogenic changes resulting from infection with other viruses studied resemble the changes seen with poliovirus, but usually progress at a slower rate.

The susceptibility of amnion cells to various viruses has been studied (Table I). All strains of poliovirus tested produced infec-

TABLE I. Susceptibility of Human Amnion Tissue Cultures to Virus Infections as Reflected by Cytopathogenic Changes, Recorded on 5th or 6th Day.

Poliovirus	Type I		4+
	II		4+
	III		4+
*Theiler's mouse poliovirus (TO-6)			0
†APC virus, Huebner-Hilleman			
	Type I	(Adenoid 71)	4+
	II	(Adenoid 6)	4+
	III	("GB")	4+
	IV	(RI 67)	4+
	V	(Adenoid 75)	4+
	VI	(Tonsil 99)	4+
	VII	(Gomen)	4+
	VIII	(Trimborn)	3+
	Simian I	(Bertha)	4+
	II	(Hull, WV)	4+
*Herpes simplex			4+
*Newcastle disease			0
*Western equine encephalitis		(8J-14)	2+
*Encephalomyocarditis virus		(MM)	0
‡Influenza	A	(GLNTC-1134)	0
*	A	(PR-8)	0
‡	B	(GLNTC-1760)	0
Coxsackie virus,	Type B-2		0
	B-3		4+
	B-4		0
	A-9		4+

0 = no change, 4+ = degeneration of almost all cells.

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† Kindly supplied by Dr. Edwin H. Lennette, State of California, Department of Public Health, Berkeley.

‡ Kindly supplied by Naval Medical Research Unit No. 4, Great Lakes.

tion and cytopathogenic changes. Sensitivity equaled that of monkey kidney, both with stock cultures of virus and stool specimens from patients. Stool "toxicity" was no more troublesome than in monkey kidney. A marked difference between monkey kidney and amnion occurred in the Coxsackie B series, the susceptibility of amnion resembling that seen with HeLa cell cultures(5,6). Only type B-3 produced cytopathogenic effects in amnion, while 2, 3, and 4 did so in monkey kidney. Types B-1 and B-5, which are known to cause degeneration in monkey kidney(7) were not tested. Of the Coxsackie A group, only type A-9 has been studied, and this produced cytopathogenic changes both in monkey kidney and in human amnion. Strains of Influenza A and B, which caused cellular changes in monkey kidney, failed to produce similar changes in amnion. Hemagglutination studies of the supernatant were not performed. Herpes simplex and Western Equine Encephalitis viruses also produced cellular degeneration in both types of tissue. No "wild" viruses have appeared in amnion cultures.

Summary. 1) A simple, reliable, and reproducible method has been developed to grow monolayer cultures of human amnion. 2) Placentas from routine vaginal deliveries are used without modifying patient preparation or care. 3) Unminced, washed amnion is trypsinized at room temperature, and the resulting cell suspension is inoculated into bottles or tubes. The best results are obtained by first growing the cells in large bottles and transferring them to tubes. 4) Susceptibility of human amnion tissue cultures to a variety of viruses has been studied, and the results are reported.

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Plasma Amino Nitrogen and Creatine Values of Growing Chick and Laying Hen.* (22536)

R. C. SALANDER AND HANS FISHER. (Introduced by G. Litwack.)
Poultry Department, Rutgers University, State University of N. J., New Brunswick.

A search of the literature reveals few reliable standard values for amino nitrogen and creatine in chicken blood. Sturkie(1) does not present any amino nitrogen values in his summary of the chemical constituents of chicken blood, nor does Albritton(2) in his monumental reference on standard blood values. Edwards and Wilson(3) reported a higher blood amino nitrogen level in the nonfasted laying hen under conditions of hyperthermy. Hsu and Combs(4) noted that a vit. B_{12} deficiency of 4-week-old chicks resulted in significantly higher blood nitrogen compounds, including amino nitrogen. There is considerably more information in the literature on creatine(1,2) than on amino nitrogen values for chicken blood, but these determinations were made with alkaline picrate and without due correction for non-creatinine compounds that give a positive Jaffe reaction.

Methods. In this laboratory plasma cre-

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TABLE I. Representative Values for Creatine and Amino Nitrogen in Chicken Blood.

	Creatine,* mg/100 ml plasma†	Amino nitrogen, mg/100 ml plasma
	5-wk-old cockerels‡	
	.83	8.74
	.92	8.52
	1.08	8.30
	.50	8.16
	.83	7.96
	.67	8.30
	1.17	7.96
	1.58	9.28
	1.08	7.20
	1.08	9.28
Avg ± S.D.	.97 ± .09	8.37 ± .40
	Laying hens	
	1.5	8.10
	1.0	7.42
	.92	8.10
	.75	8.10
	1.25	7.76
	1.83	8.40
	1.33	7.64
	.92	7.96
	1.42	8.30
	.74	7.96
Avg ± S.D.	1.17 ± .13	7.97 ± .09

* Measured as total creatinine; preformed creatinine was not measurable.

† Assuming a hematocrit of 30%, plasma values can be converted to whole blood values by multiplying by 0.7; avg values are .69 ± .05 and .82 ± .06 for cockerels and hens respectively.

‡ Differences between chicks and hens are not significant.

atine and amino nitrogen are now being determined as part of a routine clinical procedure. Plasma creatine is determined by the method of Owen, *et al.*(5). After conversion of creatine to creatinine, Lloyd's reagent in acid solution is used to absorb selectively the plasma creatinine; subsequently, it is eluted from the silicate by alkaline picrate, thus eliminating the presence of materials that would give a positive Jaffe but false creatinine reaction. Amino nitrogen is determined by the naphthoquinone method of Danielson as presented by Hawk, *et al.*(6). Representative values from the plasma of growing chicks and laying hens are shown in Table I.

Results. Edwards and Wilson(3) using the copper method of Albanese and Irby(7) reported values of 7-12 mg of amino nitrogen per 100 ml blood. Hsu and Combs(4) employing the naphthoquinone method reported amino nitrogen values of 20-25 mg/100 ml blood. Both investigators used a Folin-Wu tungstic acid protein-free plasma filtrate for their determinations yet reported their values in terms of whole blood. The report of Hawk, *et al.*(6) that red blood cells have a significantly higher content of amino nitrogen than the plasma, emphasizes the importance of properly presenting amino nitrogen contents in terms of the specific blood fraction analyzed. Another source of error often encountered if values are presented in terms of whole blood is the hematocrit. The hematocrit for chick and hen blood (but not for adult roosters) is 28-30(8) in contrast to the value of 47-50 of man and most mammals(2). Thus, if not taken into account, the hematocrit conversion factor can involve an appreciable error. The representative values for amino nitrogen in chicken plasma presented in Table I fall within the range reported for human plasma(6). The high values reported by Hsu and Combs have not been confirmed in this laboratory. The blood creatine values reported by Sturkie(1) range from 0.8-1.8 mg/100 ml blood with only 2 of 10 values falling below 1 mg/100 ml. These values are generally higher than those found in this study in which Lloyd's reagent was employed to selectively absorb creatinine. Comparisons obtained with and without Lloyd's reagent on the same sample of plasma invariably gave significantly higher readings in the absence of Lloyd's reagent. This corroborates the findings of Owen, *et al.*(5) of the presence of non-creatinine materials in chicken and human blood that give a positive Jaffe reaction.

Summary. Representative values for amino nitrogen and creatine are given for chicken blood. Creatine values previously reported in the literature appear to be too high because of non-creatine chromogens.

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Fractions of Commercial Pitressin which Release ACTH.*‡ (22537)

W. W. SWINGLE, L. J. BRANNICK, A. F. PARLOW AND WALTER BARRETT.
Biological Laboratory, Princeton University, Princeton, N. J.

Commercial Pitressin and certain fractions derived from it by chromatography, acid hydrolysis, Craig counter current distribution and other methods, are active ACTH releasing agents(1-9). In a footnote to an earlier communication(7), the writers called attention to the marked ACTH-releasing activity of hydrolysates of pitressin (Vasopressin Injection, Parke, Davis and Co.), in which the pressor activity had been destroyed by boiling for 1.5 hours in 2.2 N HCl. In addition to the releasing factor the acid hydrolysates contain a component indistinguishable in its activity from histamine when tested on the isolated ileum of the guinea pig and blood pressure of the etherized-atropinized cat.

Methods. A simple modification of the method of Saffran, *et al.*(5,6), was employed for determining the ACTH-releasing potency of pitressin† and its various fractions obtained by acid hydrolysis and Craig counter current distribution. Halved anterior pituitaries of adult rats were incubated in the Warburg apparatus for one hour with 0.67 mg of the test material. Small amounts of the incubation fluid (0.20 cc diluted to 0.5 cc), were then infused i.v. into 120-140 g male rats hypophysectomized 18-24 hours previously for adrenal ascorbic acid assay, as a measure of the efficacy of the test material for releasing ACTH.

Results. Table I-A gives the pertinent data obtained from experiments on the ACTH-releasing substance remaining in the pitressin hydrolysates after all pressor activity had been destroyed by boiling in strong acid(7).

Hydrolyzed pitressin exhibits potent releasing activity when incubated with halved pituitary glands *in vitro*, since the fall in adrenal ascorbic acid of the hypophysectomized rats averaged well over 100 mg %. The control halves of the same pituitary glands generally released small amounts of ACTH when compared with the quantities released by the experimental series. Arterenol added to the incubation fluid as originally recommended by Saffran, *et al.*, does not enhance releasing activity (Table I,A); therefore, it was not used in subsequent experiments. The hypophysectomized rat shows no depletion of adrenal ascorbic acid when infused i.v. with 300 units of pitressin boiled in 2.2 N HCl for 1.5 hours, despite the fact such material is highly active in releasing ACTH from pituitaries incubated in the Warburg.

Histamine, equivalent to 0.22 μg of the base, when added to incubation fluid containing halved pituitary glands did not induce release of greater amounts of ACTH than the corresponding control glands. These data agree with other reports of the ineffectiveness of this amine as an ACTH releaser(3,4) and make it unlikely that the histamine-like sub-

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† We are indebted to Dr. D. A. McGinty of Parke, Davis and Co., for supplies of Pitressin.

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stance which becomes evident in acid hydrolysates of pitressin is the ACTH-releasing factor.

Fractionation of pitressin by Craig counter current distribution. Non-hydrolyzed pitressin was fractionated in the Craig apparatus

TABLE I. ACTH-Releasing Activity of Pitressin.

		Sayers assay†	
		No. of rats	Ascorbic acid depletion, mg%*
A. Release of ACTH from ant. pituitaries *in vitro* by hydrolyzed pitressin, .67 mg in .55 cc incubation fluid/Warburg flask			
	Hypophysectomized		
Pitressin—hydrolyzed		37	−111 ± 18.6‡
Control		43	− 23 ± 6.6
Pitressin—hydrolyzed; arterenol added		5	−117 ± 32.8‡
Pitressin—hydrolyzed mildly in 0.6 N HCl for 30 min.		7	−103 ± 7.0‡
Control		8	− 18 ± 8.4
Histamine, 0.22 μg		6	− 31 ± 21.8
Control		6	− 36 ± 19.5
B. Release of ACTH *in vitro* by nonhydrolyzed pitressin fractionated by Craig counter current distribution, then hydrolyzed for testing			
Fraction (0.67 mg/flask)	Distribution coeff. (K)		
A_2–A_3	.59	8	−111 ± 12.0‡
Control		7	− 34 ± 16.8
B_1–B_2	1.33	8	−111 ± 15.2‖
Control		6	− 70 ± 7.9
C	4.24	7	−100 ± 21.7‡
Control		4	− 27 ± 28.0
B_2, nonhydrolyzed	1.33	5	−133 ± 15.0‡
Control		6	− 39 ± 10.0
C. Release of ACTH *in vitro* by pitressin hydrolyzed and then fractionated by Craig distribution			
(.67 mg/flask)	K		
Hydrolysate before Craig distribution		5	− 87 ± 12.1§
Control		6	− 39 ± 10.0
Hydrolysate after Craig distribution			
Fraction #1	1.33	5	−124 ± 16.1‡
#2	.75	5	−105 ± 14.7‡
Control		7	− 4 ± 12.3
Fraction #3	.42	5	− 5 ± 13.8
#4	4.24	5	− 40 ± 11.0‡
Control		4	− 29 ± 5.6
D. Release of ACTH *in vivo* by nonhydrolyzed pitressin in steroid-inhibited, intact rats			
	Dose, i.v.	Intact	
Pitressin nonhydrolyzed	3 units/rat	7	− 98 ± 8.4
Idem	5 "	5	−104 ± 19.1
Control (saline)	0.5 ml/rat	15	− 7 ± 12.8
E. Non-effect of i.v. injections of pitressin hydrolysate on adrenal ascorbic acid of hypophysectomized rats			
Pitressin—hydrolyzed	300 units/rat	8	− 8 ± 12.8

* Mean stand. error.

† Each rat intravenously infused with equivalent of .20 cc of incubation fluid from a Warburg flask containing 5 mg of anterior pituitary tissue.

‡ Differs significantly from controls ($P < .01$).

§ *Idem* ($P < .02$).

‖ " ($P < .05$).

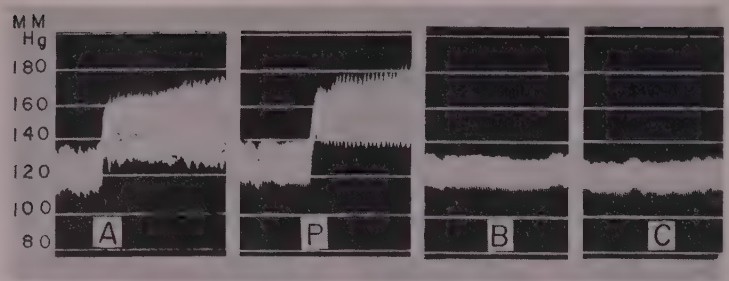

FIG. 1. Isolation of pressor factor in one fraction of pitressin by Craig counter current distribution. Dog, 11.4 kg, nembutal anesthesia, carotid blood pressure. A: Fraction A 0.05 unit/kg i.v. P: pitressin 0.05 unit/kg i.v. Fraction B: 1.0 unit/kg i.v. Fraction C; 1 unit/kg i.v. Fractions A, B and C are all active ACTH-releasing agents.

using secondary butanol and a 0.5% solution of trichloroacetic acid as a solvent system.§ One hundred transfers were made and the resulting fractions grouped on the basis of their distribution coefficients (Table I,B). Fractions A_2-A_3 administered i.v. to an anesthetized dog induced a sharp rise in the arterial pressure characteristic of the pressor action of commercial pitressin (Fig. 1). Fractions B and C were without effect on the blood pressure even in large doses, indicating that all of the pressor activity is confined to the A fraction. All fractions except B_3 were then hydrolyzed as usual and tested for ACTH-releasing activity. It should be noted, however, that the A, B, and C fractions all possessed ACTH-releasing activity, indicating that although the Craig counter current procedure, using the solvent system employed, was effective in concentrating the pressor factor in fraction A, it failed to isolate the releasing agent, which remained widely distributed. It was not difficult to rid the pitressin of all pressor activity by employing (1) strong acid hydrolysis; and (2) distribution in the Craig apparatus, but it remained to be demonstrated that the ACTH-releaser was definitely not the histamine-like component of the hydrolysate reported in earlier communications to be present in such material(7,8). Substantial evidence for complete separation of these activities was secured as follows: pitressin was hydrolyzed and then tested for ACTH-releasing activity by the *in vitro* Saffran technic and Sayers test and found to be active (Table I,C). The same material when tested on the isolated guinea pig ileum elicited typical histamine-like responses. The hydrolyzed pitressin was then distributed in the Craig apparatus and the various fractions tested by the usual method for determining ACTH-releasing activity and histamine-like response. Two fractions (Table I,C) of the hydrolyzed, Craig-distributed material were potent and resulted in sharp decline in the adrenal ascorbic acid of -124 ± 16.1 and -105 ± 14.7 mg %. One fraction, No. 4, was less potent and another fraction, No. 3, was completely inactive. However, negative results were obtained in repeated tests of the same material, both active and inactive as ACTH releasers, when tested on the guinea pig ileum. Thus, significant depletion of adrenal ascorbic acid occurs in hypophysectomized rats when these animals are infused with Warburg fluid obtained from incubation of halved anterior pituitaries with pitressin from which the histamine-like component has been removed by counter current distribution.

The corticotropin-releasing action of non-hydrolyzed pitressin can be readily demonstrated *in vivo* by i.v. infusion of 3-5 units into intact rats whose ACTH output has been inhibited by subcutaneous injections 16-20 hours previously of 10 mg each of microcrystalline DCA and the free alcohol of hydrocortisone suspended in 15% ethanol and Tween 80. Significant decline (98-104 mg %)

§ We are indebted to the Merck, Sharp and Dohme Research Laboratories for the counter current distributions recorded in this paper.

in adrenal ascorbic acid occurred in the experimental series whereas saline injected controls gave negative results (Table I,D), thus confirming the work of Porter and Rumsfeld (14).

Discussion. It is evident(1-9) that commercial pitressin contains a fraction active in releasing ACTH. The exact nature of this substance has not been definitely established although there is some evidence(3-6) that it is a peptide. It is apparent that the pressor factor in pitressin can be concentrated in one fraction by Craig counter current distribution. However, other fractions lacking the pressor agent retain their ACTH-releasing potency as do pitressin hydrolysates in which all pressor activity has been destroyed. The histamine-like component of commercial pitressin which appears in the acid hydrolysates is not to be confused with the ACTH releaser, since it, too, can be separated from the latter by counter current distribution.

It was recently announced(4,8) that Substance P, the smooth muscle stimulating factor originally described by von Euler and Gaddum(10), is an active ACTH-releasing agent. Data obtained by Guillemin and the writers afford evidence that the releasing factor in Substance P is a different entity from that commonly designated as P and can be separated from it by chromatography(4,9). It seems not improbable that the ACTH-releasing agents associated with both commercial pitressin and Substance P are identical substances. It is interesting to note that both occur in appreciable quantities in the hypothalamus and that Substance P is widely distributed throughout the nervous system(11, 12,13).

Summary. Commercial pitressin, hydrolyzed or nonhydrolyzed, exhibits ACTH-releasing activity when incubated with pituitaries in Warburg flasks and the incubation fluid infused i.v. into hypophysectomized rats. The ACTH-releasing agent of pitressin can be separated from the pressor component by Craig counter current distribution or by destroying the latter by acid hydrolysis. The hydrolysates when infused i.v. do not deplete the adrenal ascorbic acid of hypophysectomized rats. Unmodified pitressin will release ACTH *in vivo* from pituitaries of steroid inhibited rats. The histamine-like substance present in acid hydrolysates is not the ACTH-releasing agent and can be separated from it. Histamine added to the Warburg incubation fluid does not induce ACTH release.

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Recovery of Cytopathogenic Agent from Chimpanzees with Coryza. (22538)

J. A. Morris, R. E. Blount, Jr. and R. E. Savage. (Introduced by J. E. Smadel.)
Department of Virus Diseases, Walter Reed Army Institute of Research, Washington, D.C.

During October, 1955, a respiratory illness characterized by coughing, sneezing and mucopurulent nasal discharge occurred in a colony of 20 "normal" chimpanzees at the Walter Reed Army Institute of Research. The present paper describes the isolation of a virus of apparent etiologic significance in the epizootic, establishes an etiologic association between the chimpanzee coryza agent and respiratory illness in a laboratory worker and finally, presents serologic data suggesting that a number of human beings have experienced infection with the chimpanzee coryza virus or an agent closely related to it.

Materials and methods. Chimpanzees and collection of specimens. The chimpanzees in the epizootic were 15 to 20 months old and were obtained from a commercial breeder in Dania, Fla., 3 to 24 weeks prior to their illness. They were housed at the Forest Glen Annex of the Walter Reed Army Institute of Research (WRAIR). Blood specimens for serological study were obtained from individual chimpanzees at outset of the epizootic on Oct. 13, 1955, when 5 of the 20 animals were suffering from clinical coryza, and periodically thereafter until Apr. 18, 1956. Throat swabs were obtained from all animals in the colony on Oct. 17, 1955 when 14 of the 20 animals were clinically ill with coryza; the swabs provided the material employed for viral isolation studies. Another group of somewhat older chimpanzees was used in studying experimental transmissibility of the coryza. The 6 animals in this group had been inoculated previously with material presumed to contain the virus of human infectious hepatitis; they were housed in a different location from the others and had had no direct contact with other chimpanzees for over a year. *Tissue cultures.* Cultures of epithelial-like cells derived from human liver (Chang strain) were prepared by the method of Chang(1). The cultures were grown in roller tubes (1.5 x 13 cm) and stationary bottles (4 x 4 x 14 cm) in nutrient medium consisting of 8 parts Eagle's basal medium(2), 2 parts inactivated horse serum, and 0.2 part L-glutamine. Penicillin (100 units/ml) and streptomycin (20 μg/ml) were added to control adventitious bacterial contaminants. Tubes and bottles contained 1 ml and 15 ml of nutrient fluid, respectively. The cells were fed on the 3rd or 4th day by replacing the old nutrient fluid with an equal amount of fresh nutrient. Cultures were incubated at 36°C and at the time of use were usually 4 to 6 days old. *Isolation of coryza agent.* A fresh (within the hour of collection) throat swab from a chimpanzee (Sue) involved in the epizootic was washed in 2 ml of tissue culture nutrient fluid containing antibiotics. After centrifugation at 3000 rpm for 15 minutes to remove large particles, 0.2 ml of the supernatant was inoculated into a roller tube culture of 4-day-old Chang liver cells. After 4 days incubation the original cell nutrient was replaced with fresh nutrient. Four days later cellular degeneration characterized by rounding, granulation, and sloughing from the tube wall was noted. Serial transmission of the cytopathogenic agent to other tube- or bottle-cultures of Chang liver cells was readily accomplished by passage of suspensions of degenerated cells in their infected fluids. Similar isolation attempts which were made with materials obtained on October 17th from 13 other ill chimpanzees gave negative results. *Serologic procedures. Virus.* Seed virus was obtained by inoculating bottles of liver cells with the chimpanzee coryza agent (CCA) and harvesting cells and fluids 8 days later when the infected cells characteristically showed complete degeneration. After grinding in a TenBroeck grinder the mixture was clarified by centrifugation at 3000 rpm for 15 minutes. The resulting supernatant constituted the seed virus. Infectivity was preserved by storage at −70° in sealed glass ampoules. *Neutralization tests.* Serial 2-fold

dilutions of serum which had been inactivated at 56° for 30 minutes (0.15 ml) were mixed with a constant amount of virus (100 to 1000 tissue culture LD_{50}) contained in 0.15 ml of infected tissue culture material. The mixtures were incubated in a water bath at 37°C for 1 hour after which 0.1 ml of each mixture was added to each of 2 tubes containing normal liver cells. The cultures were examined microscopically for cellular degeneration after a 6- to 8-day incubation period. The neutralization titer was considered the highest dilution of serum completely inhibiting cellular degeneration. Appropriate cell and serum controls and a virus titration were included in each test. *Complement fixation tests.* Satisfactory complement fixing antigen was prepared from infected liver cells grown in medium containing 20% inactivated horse serum. When the horse serum component of the medium was not heated at 56° for ½ hour the material was anticomplementary if used in the complement fixation (CF) procedure employing overnight fixation in the cold, in accordance with the standard technic of the Department of Virus Diseases, WRAIR (3) which was used in the current studies. For use in CF tests human and chimpanzee sera were inactivated for 30 minutes at 56°C and 60°C, respectively. The serum titer was expressed as the reciprocal of the highest dilution giving 75% or greater fixation of complement after overnight incubation at 4°C in the presence of 2 units of antigen and 2 full units of complement. Controls included in each test were antigen (prepared from uninfected liver cell cultures propagated in inactivated horse serum), positive serum (obtained from a man who experienced a laboratory infection, patient B1 in Text Fig. 1, and saline.

Results. Behavior of chimpanzee coryza agent (CCA) in liver cell culture. Inoculation of CCA obtained from the culture of the throat swab of chimpanzee Sue into liver cell cultures produced little or no change during the first 5 or 6 days. On about the 7th day scattered islands of round and granular cells appeared and a few cells were disintegrated and dislodged from the glass wall of the container. Once begun, the process of degeneration spread rapidly and within 24 hours practically all cells were dead and some were floating in the nutrient fluid. Intranuclear and intracytoplasmic inclusions which are eosinophilic in Giemsa-stained cell preparations, were observed in cultures of liver cells infected with CCA. However, similar inclusion-like bodies were demonstrated in uninoculated cells, grown in inactivated horse serum. At the present time the significance of the inclusion-like structures found in infected and uninoculated cells cannot be stated with certainty.

Pathogenicity of CCA for laboratory hosts. Tissue culture materials containing 100 to 10,000 TC LD_{50} of CCA were inoculated by the intracerebral and intraperitoneal routes into one-day-old mice, weanling hamsters and young adult rabbits and guinea pigs. Other 8 to 10 gram mice, young adult rats and 16 to 20 lb chimpanzees were inoculated intranasally. Groups of chick embryos (7 to 11 days old) were inoculated on the chorioallantoic membrane and into the amnionic, allantoic and yolk sacs. With the exception of a single guinea pig that developed persistent fever beginning on the 3rd day, none of the inoculated animals or embryonated eggs other than chimpanzees developed signs of disease during observation periods ranging up to 28 days. The etiology of fever in the guinea pig was ultimately traced to a bacterial infection. Further, the fluids obtained from chick embryos inoculated by various routes failed to agglutinate chicken and human "O" erythrocytes. Tube cultures of human cells derived from conjunctiva (Chang), intestine (Henle) and human embryo fibroblasts (obtained from Microbiological Associates) were found to be less susceptible to the cytopathogenic effect of CCA than liver cells; these cells showed only incomplete degeneration after 16 days incubation. Monkey kidney cells underwent complete degeneration 8 days after infection with CCA but the cytopathogenic effect obtained was sometimes difficult to interpret because of occasional presence in the cultures of adventitious simian viruses(4).

Relation of CCA to epizootic coryza in

TABLE I. Serological Findings with Chimpanzee Coryza Agent (CCA) and Selected Chimpanzee Sera.

Chimpanzee	Serum date	DD	Chimpanzee coryza agent CF	Chimpanzee coryza agent Neut	Antibody titers against Other agents RI-67 (CF)	Influenza (HAI) PR-8	FWI-50	FLWI-52	LEE	IB1
Sue (source of CCA)	10/13/55	−3	0*	0*	10	80	20	10	80	80
	12/ 5	50	80	10	10	40	20	10	80	80
Pug	10/13	3	0	0	10	40	20	10	20	80
	12/ 5	53	40	10	10	40	40	10	10	40

* No fixation or neutralization at 1:10 dilution of serum.

chimpanzees at Forest Glen. CCA was found to be related to the epizootic disease of chimpanzees at the Forest Glen Annex of the WRAIR by the use of serologic technics. Illustrative results of CF and neutralization tests performed on sera from 2 of the chimpanzees in the Forest Glen epizootic are shown in Table I. It is seen that in both animals CF antibody against CCA was undetectable in the early sera but titered 1:40 or 1:80 in the sera taken approximately 2 months later. During the same period there was no significant change in CF antibody titer against the RI-APC-ARD virus or in HAI antibodies for any of 5 strains of influenza virus. Finally, neutralizing antibody against CCA developed in both chimpanzees during the period between bleedings. The etiologic relation between CCA and the epizootic disease in the chimpanzees is supported further by the data obtained when sera of all 20 animals involved in the Forest Glen epizootic were examined for specific complement fixing antibody. As shown in Table II all 14 chimpanzees that experienced clinical coryza during the 3rd week of Oct., 1955, subsequently developed specific antibody. Four other animals that did not suffer clinical coryza likewise produced antibody, hence, they presumably experienced unrecognized infection. The remaining 2 animals apparently escaped infection; they neither suffered clinical disease nor developed CCA CF antibody.

TABLE II. CF Antibody Titers Obtained in Sera of Chimpanzees Involved in Coryza Epizootic.

Clinical coryza	Date serum collected	No. of chimpanzees showing antibody titer of <10	10	20	40	80
Yes	10/13/55	13		1		
(14 animals)	12/19		2	6	5	1
	1/24/56		4	5	4	
	4/18	7	3	4		
No	10/13/55	6				
(6 animals)	12/19	3	3			
	1/24/56	5		1		
	4/18	6				

Experimentally induced coryza in chimpanzees. Three chimpanzees, 20 to 24 months of age and weighing 16 to 20 lbs were inoculated intranasally on Feb. 2, 1956 with 1.0 ml of 11th passage tissue culture material containing 10,000 TC ID_{50} of CCA. At the same time 3 other chimpanzees housed in the same room were injected intranasally with an uninfected Chang liver cell preparation. Results of this experiment are presented graphically in Fig. 1. Three days (Feb. 5) after inoculation 2 of the 3 chimpanzees receiving CCA developed respiratory illnesses characterized by sneezing, coughing and subsequently mucopurulent nasal discharge. These signs increased somewhat in severity and persisted at this level for 4 to 5 days; however, the animals were not febrile. By the 14th day the affected chimpanzees were free of signs of respiratory disease. The third chimpanzee in this group (Babe in Fig. 1) remained well throughout the period of observation. Of particular interest is the finding that this animal possessed CF and neutralizing antibodies (titers 1:40 and 1:20, respectively) at the time of inoculation. Two of 3 control chimpanzees that were housed with animals inoculated with CCA also developed disease. Onset of illness in these control animals occurred on the 7th (Feb. 9) and 9th (Feb. 11) day after receiving the non-infec-

Inoculum (2/2/56)	Host	Date	February 5	7	9	11	13	15	17	19	21	23	25	27	March 6
	Chimp														
CCA, 10,000 TC LD_{50} intranasally	Clark	Clinical disease													
		CP agent recovered		+				0							
		Antibodies CF 0		0					160						160
		Neut. 0		0					20						20
	Frank	Clinical disease													
		CP agent recovered		+				0							
		Antibodies CF 0		0					160						160
		Neut. 0		0					80						80
	Babe	Clinical disease													
		CP agent recovered													
		Antibodies CF 40		40					80						80
		Neut. 20							20						10
Normal TC material (contact with chimps experimentally infected with CCA)	Beanie	Clinical disease													
		CP agent recovered		+				0							
		Antibodies CF 0		0					160						160
		Neut. 0		0					20						20
	Betsy	Clinical disease													
		CP agent recovered		+				0							
		Antibodies CF 0		0					0				20		20
		Neut. 0		0					10						20
	Blondy	Clinical disease													
		CP agent recovered						+							
		Antibodies CF 0		0					0						20
		Neut. 0		0					10						10
None (contact with chimps experimentally infected with CCA)	Patient B1	Clinical disease													
		CP agent recovered							0						
		Antibodies CF		0							80				80
		Neut.		0							40				40

FIG. 1. Experimentally induced coryza in chimpanzees and laboratory infection in chimpanzees and man. 0 = No fixation or neutralization at 1:10 dilution of serum, the lowest dilution tested.

tious cell cultures, or 4 and 6 days, respectively, after the test animals had first exhibited symptoms. None of these chimpanzees developed fever.

From each of the 4 chimpanzees that developed obvious respiratory illness, *i.e.*, 2 test and 2 control animals, an agent cytopathogenic for liver cells was recovered from throat swabs taken on Feb. 8. In addition, a cytopathogenic agent was recovered on Feb. 15 from throat materials from the third chimpanzee receiving non-infectious materials. This animal, (Blondy in Fig. 1) did not show recognizable respiratory disease; nevertheless she developed complement fixing and neutralizing antibodies in minimum amounts. Each of the 5 recovered agents was shown to be similar to or identical with CCA in neutralization tests with specific antisera prepared in rabbits against the Sue strain and in complement fixation tests with a human serum that was known to react with CCA antigen.

It may be mentioned here that the 6 chimpanzees in this experiment (Fig. 1) were challenged by the intranasal instillation of 1 ml of tissue culture material containing 1000 TC LD_{50} of CCA on March 28, 1956, 55 days after the original exposure when each animal possessed demonstrable CF (range 1:20 to 1:160) and neutralizing (range 1:10 to 1:20) antibodies. All 6 chimpanzees remained free of clinical evidence of disease during an observation period of more than a month; moreover, during the same 30-day period there was no appreciable change in serum antibody titers.

Infection of laboratory worker with CCA. During the second week of February 1956, an illness diagnosed clinically as "upper respiratory infection" occurred in a laboratory worker who was working with CCA and who had had intimate contact with the experimentally infected chimpanzees. His illness was characterized by several days of nasal

TABLE III. CCA Complement Fixing Antibody in Persons of Different Ages.

Age groups (yr)	No. sera Tested	Positive	Reciprocal of CF titer
½– 2*	12	1	40
3– 6*	12	0	
7– 9*	9	0	
10–14*	12	2	20, 10
15–18*	13	3	20 for all 3
18†	40	8	80, 40, 20, remainder 10

* Sera from patients with non-respiratory illnesses submitted for diagnostic studies to WRAIR.
† Enlisted personnel, WRAMC, Mar. 1956.

snuffiness, rhinorrhea, cough, malaise, followed by several days of low grade fever and frontal headache. CF and neutralizing antibodies against CCA were undetectable in the sera of the patient taken on Feb. 8, 1956, but titered 1:80 on Feb. 22, 1956. The single attempt to recover a cytopathogenic agent from throat washings taken on the 6th day of this man's illness was not successful. The serologic findings which are shown graphically in Fig. 1 (Patient B1) are taken as presumptive evidence that the CCA was of etiologic significance in the patient's illness.

Serologic reaction of CCA with sera obtained from animals immunized against different viruses. Sera obtained from animals immunized against a variety of viruses were examined in complement fixation and neutralizing tests for their ability to react with CCA. The antisera included those prepared in monkeys against the Enders strain of measles virus*(5), Chanock's croup virus*(6) and Sabin's chimpanzee rhinitis 1954 virus*(7); in rabbits against several strains of Coxsackie virus, (Group A, type 9 and Group B, types 1, 2, 3 and 4), certain "orphan" viruses(8) [Walter Reed prototypes 7043 (untyped), 7045 (ECHO type 6) and 7054 (ECHO type 2)] and simian virus SV_5(4) and in chickens against simian virus SV_{59}(4).) All these antisera failed to react with CCA in complement fixation or neutralization tests.

Occurrence of antibody against CCA in human sera. Results of CF tests to determine the occurrence of CCA antibody in different age groups in the human population are given in Table III. The sera were obtained from patients at the Walter Reed Army Medical Center with a variety of illnesses. It is evident from the tabular data that a number of human beings possessed CF antibodies that react with CCA antigen. Furthermore, such antibodies were uncommon in children but were present in about 20% of the persons in the small group of adolescents and young adults examined. It is of some interest that the 40 young adults listed in the table were barrack mates of patient B1. Paired sera from groups of patients with common cold, bronchitis, cold agglutinin positive primary atypical pneumonia, and RI-APC-ARD infection (3 pairs in each category) were tested for complement fixing antibody against CCA. Certain of these tests were performed by Dr. Sidney Katz in Dr. John Dingle's laboratory in Cleveland using their sera and antigen supplied by us. None of the patients displayed a significant increase in CCA antibody. Nevertheless, certain of the patients possessed throughout their illnesses constant amounts of CCA antibody with titers ranging up to 1:80.

Summary. A virus was recovered from throat materials of a chimpanzee with coryza during an epizootic of respiratory disease in a colony of these animals. The new agent produced degenerative changes in tissue culture, but was not pathogenic for common laboratory animals. The donor chimpanzee as well as other chimpanzees involved in the epizootic developed specific antibodies against the coryza agent during the months following the outbreak. Susceptible chimpanzees following intranasal instillation of tissue culture materials infected with the coryza agent developed clinical coryza and subsequently made specific antibody. A presumptive etiologic association was established between the new agent and respiratory illness in a laboratory worker, but has not been implicated in the illnesses of small groups of patients with several common types of respiratory disease. However, a number of human beings, particu-

* We are indebted to Drs. John F. Enders, R. M. Chanock and A. B. Sabin for these sera.

larly adolescents and young adults, have antibodies in their sera directed against the coryza agent suggesting that these individuals have experienced infection with the new agent or one closely related to it.

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Long-Term Radiation of Bone Following Administration of C^{14}-Bicarbonate.* (22539)

HOWARD E. SKIPPER, LINDA SIMPSON, AND MARTELIA BELL.
Kettering-Meyer Laboratory (Affiliated with Sloan-Kettering Institute), Southern Research Institute, Birmingham, Ala.

Studies on distribution, turnover, and long-term retention of C^{14} (from $C^{14}O_3$) in animal tissues have indicated that from the standpoint of C^{14} radiation hazard, the bone is perhaps of greatest concern(1-5). For several months after injection of 18 μc of $NaHC^{14}O_3$ (a 50 mc man-equivalent), the radiation doses in "active" areas of long bones of mice were greater than the "tolerated dose limit" of 0.05 roentgen equivalent physical (rep) per day. Although it is apparent that one cannot extrapolate directly from animal results to man without some quantitative results on the latter, it seems important to obtain rather extensive data with regard to long-term retention of C^{14} in bones of animals and to estimate radiation being received by the most active areas of such bones. Such data might be of value in rough approximation of the hazard involved in use of C^{14}, and with availability of some human data correlations could be made.

The present paper is a brief report extending and confirming earlier reports from this laboratory(2,3) on long-term retention of C^{14} from bicarbonate by long bones of mice and appropriate radiation calculations.

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Experimental. Each of a group of adult CFW strain mice (3 months of age) was administered intraperitoneally 100 μc of C^{14}-bicarbonate. These animals were sacrificed as indicated in Table I. A femur and humerus were taken from each animal for oxidation and activity assay in a gas phase Geiger counter(6) which had been calibrated against a Bureau of Standards $BaC^{14}O_3$ standard and shown to give results extremely close to the absolute C^{14} content. Corresponding bones from the same animals were fixed in alcohol, embedded in plastic, and ground to provide the desired cross section, and subsequently autoradiogrammed on No-Screen X-ray film. The "active" volume of each bone was calculated from the individual autoradiograms. The average length and width of the parallel autoradiogram lines were measured with a filar micrometer and the "active" bone volume calculated using the procedure and assumptions previously described (3). Based on the total activity determinations on corresponding bones of the same mouse, calculations have been made of the total activity per cmm of "active" bone and the degree of radiation (rep) in "active" bone. Results of these efforts are presented in Table I along with previously reported results(3).

TABLE I. Calculations on Radiation Received by "Active" Bone at Periods following a Single Injection of C^{14}-Bicarbonate (100 μc/Mouse).

Period after inj.	Bone	Specific activity (μc/mole C)	Avg radiation in "active" bone (rep/day) New J.B.C. 189 data	159, 1951*
1 wk	Femur	1.5	.6	
	Humerus	1.2	.4	
2 "	Femur	1.5*		.9
1 mo	Femur	1.0	.2	
	Humerus	.8	.2	
4 "	Femur	.5	.1	
	Humerus	.5	.2	
6 "	Femur	.6*		.2
	"	.5*		.2
	"	.4*		.2
8 "	Femur	.5		
	Humerus	.3	.08	
12 "	Tibia	.2		
	Humerus	.1	.03	
17 "	Tibia	.03	.008	
21 "	Tibia and humerus	.02	.005	

* Previously reported values corrected ($\times$ 5.5) because of differences in level of C^{14} inj. (18 μc *vs* 100 μc) included for comparison with present data.

These results are plotted in Fig. 1. No gross evidence of bone damage was observed in any of the animals employed in this investigation.

Discussion. It can be seen from the data presented in Table I that the specific activity of bones from mice injected with a single dose of 100 μc of C^{14}-bicarbonate drops continuously with time, until at 21 months the activity is extremely low (0.02 μc/mole of carbon, ca. 1% of the bone specific activity at one week). Agreement of the present data with previously published data is good. After injection of 100 μc (a 280 millicurie man-equivalent) approximately one year was required for the radiation being received by the "active area" of the bone to drop to the maximum tolerated level for man (0.05 rep/day). This should not be construed as evidence that C^{14} is a particularly hazardous isotope since

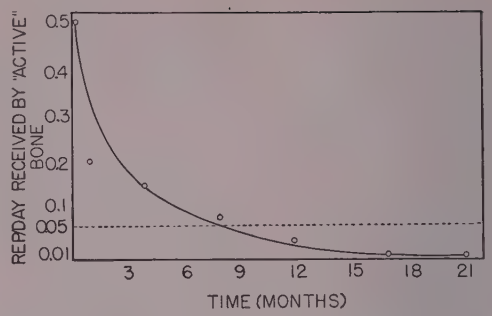

FIG. 1. Calculated radiation received by area of bone where C^{14} from $NaHC^{14}O_3$ has localized at varying periods following single inj. of 100 μc (a 280 mc man-equivalent). Dotted line indicates maximum permissible daily dose.

it is difficult to visualize accidental inhalation of millicurie quantities of $C^{14}O_2$ in any ordinary laboratory operation.

These data would seem to suggest that, for specially authorized human experimentation (in the instance of compounds in which the biological fate of the carbon isotope is $C^{14}O_2$), the present allowable doses of C^{14} are not too high.

Summary. Studies have been carried out on retention of C^{14} from labeled bicarbonate in the bones of mice. A single dose of 100 μc per mouse (a 280 millicurie man-equivalent) provided daily radiation of the most active areas of the long bones of above 0.05 rep/day for a period of approximately one year.

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Propagation of Theiler's Virus in Murine Ascitic Tumors.* (22540)

HAROLD E. PEARSON AND DOROTHY L. LAGERBORG.
Department of Public Health, University of Southern California School of Medicine and the Laboratory Division, Los Angeles County Hospital, Los Angeles.

Theiler's GD VII strain of mouse encephalomyelitis virus can be propagated in certain murine nervous tissue tumors but not in others(1). Sanders(2) has reported growth of this virus in ascitic forms of Bashford and Krebs carcinoma and in the S37 sarcoma. The present report gives the results of attempts to grow the virus in the latter tumors, the Ehrlich carcinoma, and in the ascitic form of two CNS murine tumors. Preliminary data were given elsewhere(3).

Methods. Virus passed more than 50 times in tissue cultures of newborn mouse brain was used. A mouse passage strain kindly sent by Dr. F. Rasmussen, U.C.L.A., also was used in some experiments. The Bashford 63 carcinoma and the Krebs 2 carcinoma passed in albino mice were kindly furnished by Dr. K. Sugiura, Sloan-Kettering Institute for Cancer Research. The sarcoma S37 was sent by Dr. J. L. Hartwell, National Cancer Institute, and was maintained in C57 mice. The ependymoma passed in albino A mice and the astrocytoma of C3H mice were those used previously(1). The Ehrlich carcinoma passed in albino mice was obtained from Dr. J. B. Field, of the Univ. of Southern Calif.

Virus was assayed by hemagglutination using serial 2-fold dilutions of virus with 0.5% human RBC. The endpoint was read from the sediment. Virus was also detected by intracerebral injection in mice with 0.03 ml of serial 10-fold dilutions of material tested. Ascitic forms of all tumors used were obtained by a method similar to that described by Klein and Klein(4). Intraperitoneal injections were made with tumor tissue broken into small fragments by passage through a perforated metal disc (1 mm holes) and a stainless steel screen (80 mesh). The tissue obtained was further broken up by suspension in Simms' solution with vigorous pipetting. After the larger clumps of cells were allowed to settle, the supernatant fluid contained small clumps of cells and single cells. The Krebs tumor was used only in solid form.

Solid forms of the tumors developed subcutaneously to approximately the size of 1 cm were inoculated, *in vivo*, with 0.01 to 0.03 ml of virus (100 intracerebral MLD_{50}); one week later the supernatant fluid from the centrifuged ground suspension of tumor was tested for viral content by intracerebral injection of mice. Minced tumor tissue mixed with virus was implanted subcutaneously in mice. The tumors developed after 7-14 days were likewise tested for virus. Minced tumor tissue mixed with virus was implanted to the chorio-allantoic membranes of 10-12-day chick embryos. After incubation of the eggs at 35°C for 8-10 days the amount of virus in the tumor tissue was determined by injection of mice.

Tissue cultures of tumor and virus were made in 50 ml stoppered Erlenmeyer flasks containing 3 ml of various media. After incubation for 2-3 days at 35°C the contents of 3 flasks were pooled, the tissue was sedimented and the supernatant fluid was tested for virus content. Cultures were also incubated for 7 days and tested for virus.

Mice with developed ascitic tumors were injected intraperitoneally with virus (100 MLD_{50}); 4 to 8 days later the ascitic cells were separated from the ascitic fluid, ground in a mortar with alundum and saline. After sedimentation of the debris, the saline supernate was tested for virus.

Results. No virus multiplication was found with Ehrlich, Krebs, Bashford or S37 tumors grown in mice and inoculated with virus or in tumor tissue inoculated with virus and grown in mice or in eggs. No virus proliferation was detected in tissue cultures of the above tumors in Simms' solution, lactal-

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bumin hydrolysate-yeast extract medium with 10% horse serum(5) or with Simms' and ST199(6) in a 1:1 mixture. Virus grows readily in all these media containing minced newborn mouse brain. Mouse ascitic fluid successfully used in tissue culture by Sanders (2) could not be used, because as obtained from our stock mice it neutralized the virus. A mouse passage strain as well as a tissue culture passage strain of virus was used for the above experiments. It was reported previously(1) that Theiler's virus can be grown in solid tumors in mice as well as in tissue cultures of astrocytoma and ependymoma. In the present study ascitic forms of both tumors were developed. Both tumors produced a markedly bloody exudate and both had a tendency to form solid tumors on the peritoneum. Two to 3 weeks after they received an intraperitoneal implantation mice were found to have 3-7 ml of ascitic fluid which contained small and large clumps of cells in addition to approximately 5-14 million single cells per ml for ependymoma and 50-100 million for the astrocytoma. Virus grew readily to attain titers of 10^{-3}-10^{-4} in tissue cultures of such cells. Virus grew when injected intraperitoneally with ascitic astrocytoma cells and grew readily in the ascitic ependymoma if the latter tumor cells were first washed free of ascitic fluid and were left in contact with virus for 1½ hr in the refrigerator (4-6°C) before the cells were passed to the peritoneal cavity of a new mouse; virus could be recovered from the washed, ground tumor cells removed 2 weeks later from the mouse. The titer of virus obtained was 10^{-4} with ependymoma and 10^{-6} with astrocytoma, taking as 10° the dilution of virus in saline used to resuspend the ground cells in a volume equal to that of the removed ascitic fluid. No virus proliferation was found by this procedure with ascitic forms of Bashford or Sarcoma 37 tumors. Moreover, no tissue culture (mouse brain) virus was adsorbed from Simms' solution by washed cell suspensions of any of the 3 carcinomas or the sarcoma left in contact with virus for 1½ hr at 4-6° or at 37°C. Virus was adsorbed under these conditions by minced mouse brain and by ependymoma tissue but not by astrocytoma.

The ependymoma ordinarily grew readily in solid or in ascitic form in Albino A mice. It could not be grown in solid form for more than the initial passage in another inbred strain of albino mice [Albino 2 strain(1)] maintained in this laboratory. One of 15 attempts to pass the tumor in ascitic form in Albino 2 mice succeeded. This ascitic tumor has now been maintained for more than 30 passages. Starting with the 6th passage it changed in character in the following respects from the ascitic tumor maintained in Albino A mice: a. The cells never formed solid tumors on the peritoneal surface, neither did they grow as solid tumors when subcutaneously implanted in either strain of mice. b. There were about the same number of tumor cells found in the ascitic fluid that would be found in A mice but almost all the cells were single cells. c. When passed intraperitoneally in A mice the cells grew in the manner just described. d. There were usually few RBC in the exudate. e. The cells did not support growth of Theiler's virus when tested in tissue cultures. f. The cells appeared histologically identical with cells seen in the A mice when examined unstained or stained with H. E. or Giemsa stains. There was no marked difference in ploidy observed in the 2 cell lines although numerical distribution frequencies for the chromosomes were not determined. Evidently this strain of cells selected or produced as a mutant by passage in Albino 2 mice is a variant which differs biologically from the Albino A passage cells or whose presence was masked in the latter environment.

Discussion. The results obtained in this study suggest that Theiler's GD VII virus is not readily propagated in Bashford, Krebs, or Ehrlich carcinomas or in the S37 sarcoma. Why this should differ from the experience of Sanders(2) is not clear. The ascitic forms of astrocytoma and ependymoma which have been developed would be expected to support growth of virus since the parent tissue did so. Although tissue cultures of the solid form of astrocytoma did not yield as much virus as did newborn mouse brain(1), the ascitic cells

did. It has not been determined whether this is a result of the presence of a greater number of intact cells present in a given weight of ascitic cells than in a solid tumor minced or whether there has been a mutant line of cells developed during routine tumor passage which yields more virus. This is under study. The development of a variant strain of ependymoma cells by passage in a partially susceptible strain of mice is of interest in that it has provided a line of cells for further study which is insusceptible to virus and which lacks the capacity to invade host tissue in the sense that it does not establish itself on the peritoneum or in subcutaneous tissue. The capacity of washed ascitic cells to become infected with virus and to propagate it in a virus-inhibitory environment, the ascitic fluid, is of interest. It remains to be determined if the virus can persist in subsequent generations of the cells. This condition might be analogous to the lysogenic bacteria-bacteriophage relationship. Such a condition has been suggested as possible for psittacosis virus (7) and for APC viruses(8).

Summary. Ascitic forms of murine astrocytoma and ependymoma have been developed. These support growth of Theiler's GD VII virus, *in vitro,* and under suitable conditions, *in vivo.* A variant strain of ependymoma, not susceptible to virus was obtained by passage of tumor in a partially tumor-resistant strain of mice. Ehrlich, Bashford 63 and Krebs 2 carcinomas and S37 sarcoma were not susceptible to Theiler's GD VII virus, *in vitro,* or in mice.

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Effect of 36-Hour Period of Pteroylglutamic Acid Deficiency on Fetal Development in the Rat.* (22541)

MARJORIE M. NELSON, HOWARD V. WRIGHT, CATHERINE D. C. BAIRD, AND HERBERT M. EVANS.

Institute of Experimental Biology, University of California, Berkeley.

Nelson *et al.*(1) have shown that a transitory period of pteroylglutamic acid (PGA) deficiency of only 48 or 72 hours during the second week of pregnancy markedly affected fetal development in the rat and invariably resulted in a high incidence of abnormal young or fetal death. The earlier phases of embryonic development were more severely affected than the later phases by either period of deficiency. In contrast, a 24-hour period of the PGA-deficient diet during the same week of pregnancy had little or no effect. In order to determine more accurately the length of time required for the PGA-deficient diet to affect fetal development and also to find the period of greatest sensitivity, the effects of a 36-hour period of deficiency have been studied.

Methods. The experimental procedures and diets were the same as those used previously to study the effects of transitory PGA-deficiency(1). Stock female rats of the Long-Evans strain were bred with normal males and given the PGA-deficient diet† for 36 hours, beginning with the 7th, 8th, 9th, and 10th days of pregnancy. Following the period of transitory deficiency, the animals were given the PGA-supplemented diet† for the remainder of the gestation period. Twenty to 25 rats were used for each experimental group. Additional groups of animals were subjected to 24- and 48-hour periods of deficiency instituted on the same days in order to confirm the results previously obtained with smaller groups for some of these periods.‡ All young were removed by cesarian section on the 21st day of gestation and examined macroscopically for abnormalities; the uterus was checked for the presence of resorbing sites.

Results. Table I summarizes the effects of 24, 36, and 48-hour periods of PGA-deficiency. A 48-hour period of deficiency started on either the 7th or 8th days of pregnancy was equally deleterious to fetal development as 97 to 100% of the embryos resorbed in both groups. When the same period of deficiency was started on the 9th or 10th days fewer embryos resorbed, 61% and 19%, respectively. Abnormal young and even a few normal young were found, showing that fetal sensitivity to PGA-deficiency decreased with increasing fetal age and differentiation. A 24-hour period of deficiency during the same days of pregnancy was, however, virtually ineffective. Only 10 to 11% of the embryos were affected, the percentage of fetal death being slightly higher than that observed in more than 40 control litters. Half of the control pregnancies had received the PGA-supplemented diet and the remainder a stock diet of natural foodstuffs. No abnormal young were present in the control litters whereas a few congenital anomalies were ob-

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† The PGA-deficient diet containing 1% succinylsulfathiazole and 0.5% x-methyl-PGA was the same as that used previously (Nelson *et al.* 1955). The PGA-supplemented diet contained the identical constituents, including the x-methyl-PGA for more than half the animals, but in addition, 50.5 mg synthetic PGA per kilo of diet. Lot N-125 of x-methyl-PGA was used throughout this study.

‡ No additional animals were subjected to the 48-hour deficiency period from days 9 to 11 or days 10 to 12. The data for these periods in Table I are taken from Nelson *et al.*(1).

TABLE I. Effect of Transitory PGA Deficiency on Fetal Development in the Rat (24, 36, and 48 Hr Periods).

PGA-deficient diet		No. rats bred	Wt change during gestation, g	Total No.	Embryos			Total affected, %
Hr	Days				Normal, %	Abnormal, %	Resorbed, %	
48	7- 9	22	+ 58	197	1	2	97	99
	8-10	23	+ 56	237	0	0	100	100
	9-11	34	+ 77	328	0	39	61	100
	10-12	21	+ 90	197	19	62	19	81
36	7- 8½	22	+ 92	206	67	6	27	33
	8- 9½	26	+ 79	256	20	15	65	80
	9-10½	24	+ 78	232	69	10	21	31
	10-11½	24	+101	240	92	0	8	8
24	7- 8	23	+109	217	90	0	10	10
	8- 9	23	+115	239	89	5	6	11
	9-10	21	+102	202	90	3	7	10
	10-11	23	+108	235	90	0	10	10
	None	42	+114	410	0	0	5	5

served in two groups subjected to 24-hour periods of deficiency. In contrast to the 24-hour periods of deficiency, the 36-hour periods markedly affected fetal development when instituted on the 7th, 8th, or 9th days. The absence of effects when the deficiency was started on the 10th day again demonstrated the rapidly decreasing sensitivity to this vitamin deficiency with increasing fetal age. The effects of the 36-hour period were greatest when the PGA-deficient diet was started on the 8th day, 80% of the embryos being affected. Approximately 30% of the embryos were dead or abnormal when the deficient diet was started one day earlier or later.

The types of abnormalities observed in the young resulting from 36-hour periods of deficiency instituted on the 7th or 9th days were similar to those previously observed for the corresponding 48-hour periods of deficiency(1). Cerebral and eye defects, cardiovascular anomalies, and gastroschisis were observed in the young from the day 7 to 8½ group; whereas cleft palate, harelip, syndactylism, clubfoot, cardiovascular and eye defects were observed in the young from the day 9 to 10½ group. In the abnormal young from the day 8 to 9½ group cardiovascular defects predominated; hydrocephalus, diaphragmatic defects, and gastroschisis were also found.

Discussion. The data presented demonstrate that fetal development in the rat can be severely injured by as brief a period as 36 hours of the PGA-deficient diet. The proportion of embryos affected varied with the time of instituting the deficient diet and was highest when the diet was started on the 8th day. The deleterious effects of the PGA-deficient diet given from days 8 to 9½ were presumably exerted during the 9th day (inasmuch as the first 24 hours were ineffective and we must assume that a few hours would be required for vitamin supplementation to counteract the antimetabolite, x-methyl PGA). Similarly, the PGA-deficient diet given from days 7 to 8½ presumably exerted its effects during the 8th day. Thus, the 8-day rat embryo appeared to be less sensitive to this vitamin deficiency than the 9-day rat embryo.

A similar difference in apparent sensitivity of the rat embryo to low doses of irradiation has been reported by Wilson(2) and by Hicks(3). It has been suggested by Russell and Russell(4) that the cells of the 8-day rat embryo may be just as sensitive to irradiation but have a greater regulatory power. The same explanation may be valid for sensitivity to PGA-deficiency. It is also possible that the cells of the earlier embryo may have fewer biochemical reactions for which the vitamin is necessary than cells of the 3 germ layers in the 9-day rat embryo. Bieber *et al.*(5) found a similar ineffectiveness of PGA antimetabolites during the early stages of development in *Rana pipiens*. Hitchings(6) has hypothe-

sized that early embryogenesis in *Rana pipiens* might occur at the expense of preformed purine-containing nucleic acid fragments stored in the ovum and that nucleic acid synthesis from smaller molecules involving PGA might begin only at neurulation, the earliest stage that PGA antimetabolites affect development in this species.

Summary. Fetal development in the rat was severely affected by a 36-hour period of pteroylglutamic acid deficiency when instituted early in the second week of pregnancy. The incidence of fetal death or abnormality was higher when the vitamin-deficient diet was started on the 8th day of pregnancy than on the 7th day but decreased rapidly thereafter with increasing fetal age.

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Vitamin E Deficiency and Biosynthesis of Glycine, Serine, and Methionine in Rabbits.* (22542)

JOHN T. SIME, DOUGLAS E. YOUNG,† JAMES S. DINNING, AND PAUL L. DAY.
Department of Biochemistry, University of Arkansas School of Medicine, Little Rock.

While studying the effect of vit. E deficiency on the incorporation of formate-C^{14} and glycine-1-C^{14} into the nucleic acids of various rabbit tissues, it was found that the deficiency also altered the level of isotope incorporation into the proteins of those tissues. The tissue protein exhibiting the greatest difference was that of skeletal muscle(1). This paper reports the results of the isolation of glycine, serine, and methionine from skeletal muscle of normal and vit. E-deficient rabbits following injections of either sodium formate-C^{14} or glycine-1-C^{14}.

Methods. New Zealand rabbits of both sexes were placed on a purified diet deficient in vit. E(2). Controls were given 4 mg of α-tocopherol acetate per kilo of body weight twice weekly. When symptoms appeared in 3-4 weeks, one deficient and one control rabbit were injected with either sodium formate-C^{14} (specific activity, 2.85 μc/mMole) or glycine-1-C^{14} (specific activity, 0.58 μc/mMole) at a level of 10 μc per 100 g of body weight. After 4 hours the animals were killed and the skeletal muscles of the hind legs were removed.

This tissue was homogenized in an equal volume of water and extracted with cold 10% trichloroacetic acid, alcohol, alcohol-ether, and hot 10% sodium chloride. The protein residue was then dried. The specific activities of these protein samples were determined by counting small aliquots in an end-window Geiger-Muller tube with window thickness of 2 mg per sq. cm. All values were corrected to infinite thinness. Approximately 10 g of the protein was hydrolyzed according to the method of Stein and Moore(3). The amino acids were separated with an ion exchange column, using Dowex 50-X4, using the method of Moore and Stein(4) with modifications. The hydrolysate was placed on the column in a citrate buffer at pH 3.1. The effluent was collected in 4 ml fractions at a rate of 50 ml per hour at 32°C. Each fraction was assayed for C^{14} content by counting an aliquot which had been evaporated to dryness.

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† Medical Student Research Fellow, National Foundation for Infantile Paralysis.

TABLE I. Incorporation of Sodium Formate-C^{14} and Glycine-1-C^{14} into Protein, Glycine, Serine, and Methionine in Skeletal Muscle from Normal and Vit. E-Deficient Rabbits.

Compound inj.	Diet	Protein, c.p.m./mg	c.p.m./μMole		
			Glycine	Serine	Methionine
Glycine-1-C^{14}	Normal	2.1	53	14	0
	Vit. E-deficient	5.6	49	13	0
Na formate-C^{14}	Normal	1.9	—	11	0
	Vit. E-deficient	11.8	—	37	41

All values were corrected to infinite thinness. The glycine content was determined by the method of Alexander *et al.*(5), serine by the method of Frisell *et al.*(6), and methionine by the method of Dubnoff(7). The specific activities were calculated for each amino acid. Two animals were used per group and the agreement was excellent.

Results. The protein from the skeletal muscle of vit. E-deficient rabbits injected with glycine-1-C^{14} contained 2.5 times as much radioactivity as the protein from similarly treated normal controls (Table I). However, the radioactivities of the glycine and serine isolated from the skeletal muscle protein of normal and vit. E-deficient rabbits were essentially the same. Since the glycine-serine interrelationship is well known, it would be expected that a major percentage of the radioactivity of any protein following an injection of glycine-1-C^{14} would be found in the glycine and serine. This does not appear to be the case in the skeletal muscle protein of vit. E-deficient rabbits. The location of the radioactivity, not accounted for in glycine and serine, is unknown. Inasmuch as only the methylene portion of glycine has been shown to be a methyl group precursor, the lack of activity found for methionine in both normal and vit. E-deficient rabbits was to be expected. The injection of sodium formate-C^{14} into normal and vit. E-deficient rabbits resulted in 6 times as much radioactivity in the skeletal muscle protein of the deficient animals. The deficiency resulted in higher specific activities for serine and methionine (Table I). These two amino acids, taken together, contain approximately the same percentage of the protein activity whether from deficient or normal animals. Considering the lack of activity in the methionine of the normal rabbits, the high specific activity of the methionine from the deficient animals is striking. This increased utilization of formate in vit. E deficiency is in agreement with previous observations. It has been reported that following formate-C^{14} injections, tissue purines (1) and urinary creatinine(8) of deficient rabbits have much higher activities than the same compounds from normal animals.

The data of formate utilization may be explained partially by the excretion of creatine and allantoin. Both compounds have been shown to be excreted in great quantity in vit. E-deficient rabbits(2,9), and the biosynthesis of each is known to involve 1-carbon fragments. Presumably, an elevated excretion is the result of an increased rate of biosynthesis which would in turn mean a decreased availability of 1-carbon fragments. Therefore, following the injection of sodium formate-C^{14}, all compounds requiring 1-carbon fragments would be found to have higher specific activities in the vitamin E-deficient rabbits than in the normal controls.

Summary. Injections of sodium formate-C^{14} resulted in no activity in the methionine from the protein of skeletal muscle from normal rabbits but such activity did appear in the methionine of vit. E-deficient rabbits. The specific activities of glycine and serine were essentially the same in normal and in deficient skeletal muscle protein following injections of glycine-1-C^{14}. In spite of this the activity of the protein from deficient rabbits was much higher than that of the normal controls.

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Effect of Intravenously Administered Amino Acids on Blood Ammonia.* (22543)

HAROLD A. HARPER, JOHN S. NAJARIAN, AND WILLIAM SILEN.
The Surgical Research Laboratories, University of California School of Medicine, San Francisco.

In a study of the metabolism of parenterally administered glycine Handler, Kamin and Harris(1) reported that glycine infused into dogs at rates exceeding 1 mg amino N/kg/min. was invariably found to be lethal, although after varying periods of time. These findings were interpreted by Handler *et al.* as a manifestation of the toxicity of glycine. Doolan *et al.*(2) in studies on renal clearance of amino acids in humans observed a severe reaction in a subject receiving a 5% solution of glycine injected intravenously at a mean rate of 0.67 mg amino N/kg/min. Because of the symptoms associated with the reaction to glycine these authors suggested that an elevation in blood ammonia might be responsible for the toxicity otherwise attributed to the amino acid itself. To investigate this possibility the effect in dogs of intravenous administration of amino acids on the levels of ammonia in the blood was studied.

Procedure. One liter of an amino acid solution was infused intravenously over a 2-hour period to adult male mongrel dogs under sodium pentobarbital anesthesia. Blood samples were drawn prior to the infusion and at 30-minute intervals thereafter. Heparin was used as the anticoagulant. Each sample was analyzed for ammonia, urea and amino acid nitrogen. Ammonia nitrogen was determined by the method of Conway(3); urea nitrogen by the method of Van Slyke and Kugel(4), and amino acid nitrogen by the method of Hamilton and Van Slyke(5).

* Supported by the Christine Breon Fund for Medical Research.

In the first series of experiments glycine was infused in doses ranging from one to 7 mg amino N/kg/min. In a similar study DL-alanine was infused at a rate of 3.5 mg amino N/kg/min. To compare the blood ammonia levels that follow the infusion of a mixture of amino acids with those after infusion of a single amino acid, an acid hydrolysate of casein fortified with DL-tryptophan† was infused at rates of 3.5 or 0.58 mg amino N/kg/min.

Results. In Table I the concentrations in blood or plasma of ammonia, urea and amino acid nitrogen after the infusion of the various amino acids are given. The data were derived from individual experiments under the conditions described. It is apparent that significant increases in blood ammonia occurred after the infusions of glycine at levels above one mg amino N/kg/min. At the maximum infusion rate used (7 mg amino N/kg/min) the dog expired in respiratory failure immediately after the 2-hour infusion. At lower rates of administration no symptoms were elicited. The elevations of ammonia in the blood were directly related to the amounts of amino acid administered, indicating an immediate relationship between the dose of amino acid and the occurrence of ammonia intoxication. The amino nitrogen levels reflect the concentration in the blood of glycine that was being infused. Conversion to urea of the nitrogen derived from the infused amino acid was responsible for the rise in blood urea nitrogen since it was proportional

† Courtesy, Winthrop Laboratories.

TABLE I. Ammonia, Urea and Amino Nitrogen in Blood at Intervals after Intravenous Infusions of Amino Acids in Dogs.

	Interval (min.) 0	30	60	90	120
			Glycine (1 mg N/kg/min.)		
Ammonia N*	.18	.52	.25	.72	.38
Urea N†	17.1	17.2 (.1)	20.4 (3.3)	21.9 (4.8)	22.4 (5.3)
Amino N‡	6.2	9.	9.5	7.1	7.6
			Glycine (1.75 mg N/kg/min.)		
Ammonia N*	.37	1.88	1.69	.63	1.55
Urea N†	25.	29. (4.)	30. (5.)	33. (8.)	33. (8.)
Amino N‡	5.1	11.7	10.6	9.7	8.1
			Glycine (3.5 mg N/kg/min.)		
Ammonia N*	.12	3.67	10.77	14.43	17.4
Urea N†	34.	36. (2.)	38. (4.)	42. (8.)	44. (10.)
Amino N‡	4.9	15.2	32.	33.7	36.7
			Glycine (7.0 mg N/kg/min.)		
Ammonia N*	.27	7.	14.9	26.3	>35.
Urea N†	16.8	19.4 (2.6)	23. (6.2)	27.9 (11.1)	32.1 (15.3)
Amino N‡	3.1	38.1	58.2	70.8	89.2
			DL-Alanine (3.5 mg N/kg/min.)		
Ammonia N*	.74	.59	1.66	1.91	1.7
Urea N†	15.7	17.6 (1.9)	22.6 (6.9)	25.6 (9.9)	27.6 (11.9)
Amino N‡	4.1	34.7	41.3	43.6	43.9
			Casein hydrolysate (3.5 mg amino N/kg/min.)		
Ammonia N*	.16	5.89	5.2	4.41	5.1
Urea N†	14.9	21.9 (7.)	26. (11.1)	31.1 (16.2)	37.8 (22.9)
Amino N‡	4.1	23.2	24.8	31.9	40.9
			Casein hydrolysate (0.58 mg amino N/kg/min.)		
Ammonia N*	.13	.55	.87	.82	.13
Urea N†	19.8	19.4 (-.4)	21. (1.2)	22.3 (2.5)	22.3 (2.5)
Amino N‡	4.6	4.6	5.5	4.4	4.6

* μg/ml.
† mg/100 ml. Increase over fasting level shown in parentheses.
‡ mg/100 ml (plasma).

to the amounts of glycine infused as well as to the blood ammonia levels.

In order to compare the effects on blood ammonia of an amino acid closely related to glycine, alanine was infused intravenously. However, because of limitations of solubility it was impractical to administer this amino acid at a rate exceeding 3.5 mg amino N/kg/min. Increases in the blood ammonia were very similar to those to be expected if one-half this dose were used, judging from the results of the glycine experiment. This suggested that the natural (L) isomer was deaminated more promptly than the D form and that the ammonia was contributed almost exclusively by the L isomer. Injections of the casein hydrolysate at a rate of 3.5 mg amino N/kg/min. also produced increases in the blood ammonia but considerably less than predicted from administration of glycine at a comparable rate. At the same time blood urea nitrogen increased over the fasting level to a greater extent than that observed when comparable amounts of amino nitrogen were given as a single amino acid. This indicated that some of the amino acids in the casein mixture exerted an influence to decrease the levels of ammonia in the blood. The concomitant rise in urea may indicate that this was effected by accelerating the rate of removal of ammonia through its conversion to urea. Administration of casein hydrolysate at a rate of 0.58 mg amino N/kg/min. corresponds to that recommended for its use under clinical circumstances. At this rate, no significant elevation of ammonia in the blood occurred.

The results of these studies indicate that

glycine infusions may be used as a means to produce ammonia intoxication for experimental purposes. This method may be more physiological than the use of inorganic compounds of ammonia.

Summary. 1. Glycine, alanine or an acid hydrolysate of casein were intravenously infused in dogs over a 2-hour period, and the concentrations of ammonia, urea and amino acid nitrogen in the blood were determined at 30-minute intervals. 2. At rates of infusion of glycine above 1 mg amino N/kg/min. significant increases in blood ammonia occurred, which were directly related to amounts of glycine infused. At the maximum infusion rate used (7 mg amino N/kg/min.) the dog expired in respiratory failure at the end of the 2-hour infusion. The results of these experiments indicate that the toxicity attributed to rapid rates of infusion of glycine may actually be due to ammonia intoxication. 3. After administration of a casein hydrolysate increases in blood ammonia were less and those of urea were greater than predicted from administration of glycine at comparable rates. This suggests that certain amino acids in the mixture decrease blood ammonia levels by enhancing urea production.

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Comparative Effect of Arginine and Monosodium Glutamate on Blood Ammonia.* (22544)

JOHN S. NAJARIAN AND HAROLD A. HARPER.

The Surgical Research Laboratories, University of California School of Medicine, San Francisco.

In a previous study(1) on the effect of intravenously administered amino acids on ammonia levels in the blood it was noted that after the administration of a mixture of amino acids (casein hydrolysate) increases in blood ammonia were less and those of urea were greater than predicted from the infusion of a single amino acid (glycine) at a comparable rate. These findings suggested that certain amino acids in the mixture effected a decrease in blood ammonia levels by enhancing urea production. The effect of a single amino acid infused together with glycine was therefore studied in order to investigate this hypothesis. For this purpose, arginine was selected because of its role in the urea cycle.

The use of monosodium glutamate has been advocated as a means to reduce high blood ammonia levels in human patients by Walshe (2) and by McDermott(3). The reduction is presumably effected through amidation of L-glutamic acid to form glutamine. It was therefore of interest to compare the effect on blood ammonia of glutamic acid with that of arginine. This would serve to indicate relative efficiency for detoxification of ammonia of amidation of glutamic acid as compared to urea production.

Procedure. Ammonia toxicity was induced in adult male mongrel dogs by intravenous administration of glycine as described by Harper *et al.*(1). Blood samples were drawn before the infusion and at 30-minute intervals thereafter, for a 2-hour period. Heparin was used as the anticoagulant. The blood samples were analyzed for ammonia, urea, and amino acid nitrogen. Ammonia nitrogen was determined by the microdiffusion method of Conway(4), urea nitrogen by the method of Van Slyke and Kugel(5) and amino acid

* Supported by the Christine Breon Fund for Medical Research.

TABLE I. Ammonia, Urea and Amino Nitrogen in Blood after Intravenous Infusions of Various Amino Acid Combinations in Dogs.

	Interval (min.) 0	30	60	90	120
	Glycine (3.5 mg N/kg/min.)				
Ammonia N*	.4	6.62	11.1	15.32	18.73
Urea N†	17.6	20.6 (3.)	24.3 (6.7)	25.4 (7.8)	27.8 (10.2)
Amino N‡	3.3	21.2	25.3	32.7	38.6
	Glycine (3.5 mg N/kg/min.) and arginine (1.75 mg amino N/kg/min.)				
Ammonia N*	.1	.99	.99	1.7	1.7
Urea N†	20.	28.2 (8.2)	38.5 (18.5)	52.5 (32.5)	63. (43.)
Amino N‡	3.7	45.9	55.3	66.1	71.5
	Glycine (3.5 mg N/kg/min.) and monosodium glutamate (1.75 mg N/kg/min.)				
Ammonia N*	.24	1.75	5.36	9.49	13.94 .
Urea N†	17.4	21.7 (4.3)	21.7 (4.3)	25.4 (8.0)	28.5 (11.1)
Amino N‡	4.6	32.3	57.8	56.6	57.1
	Glycine (3.5 mg N/kg/min.) and monosodium glutamate, 50 g during 2nd hr				
Ammonia N*	.69	6.58	11.72	19.23	24.75
Urea N†	16.9	21.9 (5.)	23.1 (6.2)	25. (8.1)	29.9 (13.)
Amino N‡	4.1	22.2	28.6	65.2	77.9
	Glycine (3.5 mg N/kg/min.) and arginine, 50 g during 2nd hr				
Ammonia N*	.55	3.59	8.81	1.29	.67
Urea N†	18.8	21.2 (2.4)	22.4 (3.6)	39.8 (21.0)	48.5 (29.7)
Amino N‡	4.2	23.3	27.6	49.4	50.3
	Glycine (3.5 mg N/kg/min.) and arginine, 25 g during 2nd hr				
Ammonia N*	.39	2.85	9.17	2.39	1.08
Urea N†	15.5	18.6 (3.1)	19.8 (4.3)	28.5 (13.)	23.5 (8.)
Amino N‡	4.6	22.8	26.5	33.3	44.6

* μg/ml.
† mg/100 ml. Increase over fasting level shown in parentheses.
‡ mg/100 ml (plasma).

nitrogen by the method of Hamilton and Van Slyke(6).

In the first series of experiments glycine was infused (1) alone, at a rate of 3.5 mg amino N/kg/min.; (2) together with 1.75 mg amino N/kg/min. of L-arginine hydrochloride or (3) with 1.75 mg amino N/kg/min. of L-monosodium glutamate. In a second series of experiments the blood ammonia was raised by the continuous infusion of glycine at 3.5 mg amino N/kg/min. for 1 hour. At this time the glycine infusion was continued at the same rate but 50 g of arginine or of monosodium glutamate were added to the glycine infusion and this mixture was administered during the second hour. A similar experiment was also conducted using glycine with 25 g of arginine added at the second hour.

Results. The concentrations in blood or plasma of ammonia, urea and amino acid nitrogen after the infusion of various amino acid combinations are shown in Table I. The data were derived from individual experiments under the conditions described. The blood ammonia levels were markedly lower when arginine and glycine were infused together as compared to those observed when only glycine was infused at a comparable rate. In fact, at the termination of the infusion of arginine together with glycine the reduction in blood ammonia was 10-fold. A striking elevation in blood urea accompanied the marked lowering of blood ammonia that was observed with arginine. On the other hand, the concomitant administration of monosodium glutamate with glycine effected only a moderate decrease in blood ammonia and the rise in urea was no greater than that expected from the amount of glycine infused. The contrast in the effects of the 2 amino acids was even more striking when the experi-

ment was conducted by attempting to lower blood ammonia after it had been allowed to increase rather than by preventing the rise through concomitant administration of the 2 amino acids. Under these conditions arginine was immediately effective in lowering the levels of ammonia in the blood whereas monosodium glutamate proved to be completely ineffective.

Discussion. The relationship between hepatic encephalopathies and elevated blood ammonia levels was first suggested by Hahn (7) in Pavlov's laboratory and later confirmed by Burchi(8) and by Van Caulaert (9). This syndrome has been actively studied in recent years because of its association with hepatic coma and its occasional occurrence as a consequence of the surgical production of vascular shunts to ameliorate portal hypertension. As a means to decrease or to prevent a rise in blood ammonia several procedures have been advocated. These include a reduction in dietary intake of protein in an effort to limit the amounts of that substrate which is the major source of ammonia produced within the intestine; oral administration of neomycin to inhibit bacterial production of ammonia within the intestine(10); and oral or intravenous administration of glutamic acid to aid in detoxification of ammonia by formation of glutamine (2,3).

Ammonia is metabolized *in vivo* by 3 routes. It may be excreted as ammonium salts, or used in transamination (*e.g.* of glutamic acid to glutamine) but it is mainly utilized in the formation of urea. Thus enhancement of urea production should be the most effective method to reduce blood ammonia. The results of these experiments indicate that arginine administration exerts an effect on production of urea as evidenced by the extensive rise in blood urea which follows its administration in the presence of excess ammonia. The effectiveness of this method to remove ammonia from the blood was demonstrated by the rapid fall which occurred in blood ammonia in as short a period as one-half hour and after an infusion of only 25 g of arginine.

duRuisseau *et al.*(11) have reported that the prior or concomitant intraperitoneal administration into rats of arginine with an $LD_{99.9}$ dose of ammonium acetate exerted marked protection against the lethal effects of ammonia intoxication. They also concluded from their studies on the levels of ammonia and urea in the blood that the ability of arginine to protect these animals was attributable to its role in production of urea.

We have applied our studies to the treatment of several patients with ammonia intoxication associated with portacaval anastomoses or with acute or chronic liver disease, with encouraging results. A report of this clinical experience is in preparation.

Summary. 1. Elevated levels of ammonia in the blood were induced in dogs by intravenous administration of glycine. 2. The effects on levels of ammonia in the blood of simultaneous or delayed administration with glycine of L-arginine hydrochloride or L-monosodium glutamate were then studied. 3. Arginine given concomitantly with glycine prevented any significant rise in blood ammonia. When administered one hour after the commencement of glycine infusion, it effected a prompt reduction of levels of ammonia in the blood. On the other hand, monosodium glutamate was only slightly effective under the former experimental conditions and completely ineffective under the latter. 4. The rise in blood urea which accompanied the marked fall in blood ammonia when arginine was given indicated that this amino acid exerted its effect on the blood ammonia by an influence on the production of urea.

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Pharmacology of the Psychotherapeutic Drug Benactyzine (β-Diethylaminoethyl Benzilate Hydrochloride). (22545)

F. M. BERGER, C. D. HENDLEY AND T. E. LYNES.
Wallace Laboratories, New Brunswick, N. J.

Several clinical studies in the European medical literature have indicated that benactyzine* (β-diethylaminoethyl benzilate hydrochloride) may be of value in symptomatic treatment of anxiety and other manifestations of psychoneurosis and psychosomatic disease (3,4,10,13,14). Jacobsen and his associates showed that the drug eliminated experimentally-induced conflict behavior in cats(7) and rats(5,8). Human beings exposed to emotion-provoking stress showed less intense autonomic responses after benactyzine(9).

Benactyzine was first prepared in 1936 (16), but studies of the pharmacological activity of the compound have been limited. *In vitro* anticholinergic and antihistaminic activity were demonstrated by Burtner and Cusic (2) using rabbit intestinal muscle. Lands *et al.*(11) showed that benactyzine has a weaker effect on salivary secretion and on dilation of the pupil than does atropine. The present study describes further pharmacological properties of the compound, and compares it with other psychotherapeutic agents.

Acute toxicity. In mice, benactyzine caused hyperexcitability and convulsions. After intraperitoneal administration of about one-half of an LD_{50} the animals became hypersensitive to sound and touch and had extended S-shaped tails (Straub reaction). Most animals also had clonic convulsions which alternated with periods of exhaustion. Doses higher than 90 mg/kg produced clonic convulsions of great violence. The LD_{50} was 155 ± 9 mg/kg and the mean convulsant dose (CD_{50}) was 86 ± 6 mg/kg. *Effect on behavior.* In adult Rhesus monkeys (*Macaca mulatta*) doses ranging from 1 to 6 mg/kg intravenously caused only minor changes in behavior. After 1 mg/kg there was mydriasis and some decrease in locomotion. Although the animal exhibited less withdrawal from the experimenter there was no real taming effect. At 2 mg/kg the animals became ataxic and occasional convulsive jerks occurred. Six mg/kg put one monkey into frank convulsions. These were clonic, lasting 1 to 5 seconds, and recurred at intervals of 5 to 15 seconds. This stage was present for about 10 minutes and was followed by post-ictal depression lasting over one hour. After intravenous administration, effects were seen within one minute and there was usually some evidence of recovery in one hour. Pupillary dilation was the most persistent effect, sometimes lasting as long as 20 hours.

Potentiation of hexobarbital anesthesia. Groups of mice were given hexobarbital sodium 100 mg/kg and benactyzine hydrochloride simultaneously by the intraperitoneal route and the duration of hexobarbital anesthesia was measured(17). The antihistaminic diphenhydramine (Benadryl), which causes marked prolongation of hexobarbital anesthesia was used for comparison. The results

* Benactyzine is the generic name for β-diethylaminoethyl benzilate hydrochloride accepted by the Scandinavian Pharmacopoeia Commission. The drug is sold in Europe under the trade names Suavitil, Nutinal and Parasan.

TABLE I. Potentiation of Hexobarbital Anesthesia by Benactyzine or Diphenhydramine in Mice. Hexobarbital soluble 100 mg/kg given intraperitoneally simultaneously with other drugs.

Drugs	mg/kg	Duration of anesthesia in mice	t	p
Hexobarbital only		29.0 ± 2.8	—	—
" and diphenhydramine	10	47.2 ± 4.2	3.6	<.01
" " "	20	67.2 ± 6.3	5.5	<.01
" " benactyzine	10	64.9 ± 4.9	6.4	<.01
" " "	20	97.3 ± 4.6	14.5	<.01

are given in Table I. Benactyzine 10 mg/kg more than doubled the duration of anesthesia produced by hexobarbital alone. Furthermore, benactyzine had a markedly stronger effect than diphenhydramine in prolonging hexobarbital anesthesia. *Effect on electroshock seizures.* These tests were carried out in mice by a technic previously described(1). The current intensity of 50 m.a. applied for 0.2 second was about 4 times the convulsive threshold dose. Most mice survived repeated shocks of this kind. After oral administration of small doses of benactyzine electroshock seizures caused death. The oral dose of benactyzine that produced death in 50% of animals after electroshock seizures was 24 ± 3 mg/kg. This dose did not produce any other observable symptom in mice except mydriasis. The latency period between administration of the electric shock and onset of the tonic extensor phase of the convulsion was unchanged after 24 mg/kg benactyzine but was significantly prolonged after 37 mg/kg.

Antispasmodic effect of benactyzine and other drugs was evaluated on the rat colon when acetylcholine and 5-hydroxytryptamine (serotonin) were used as stimulants and on the guinea pig ileum when histamine was used. The spasmogens were used in doses which produced submaximal contractions as follows: acetylcholine chloride 5 γ/100 ml, 5-hydroxytryptamine creatinine sulfate 10 γ/100 ml, histamine diphosphate 50 γ/100 ml. After several reproducible responses were obtained, the drugs were given and permitted to remain in contact with the organ for 3 minutes before addition of the spasmogen. Table II which summarizes the results of these experiments confirms the previously-reported marked anticholinergic action of benactyzine (2,11,12). In this respect the drug is about 20 times more potent than chlorpromazine. The antagonistic action of benactyzine to 5-hydroxytryptamine *in vitro* is of approximately the same order as that of chlorpromazine. Benactyzine is a relatively poor histamine antagonist being 10 times less active than chlorpromazine and 20 times less active than diphenhydramine.

Excretion of 5-hydroxyindoleacetic acid. Benactyzine in doses of 5 mg/kg did not increase excretion of 5-hydroxyindoleacetic acid in the urine of the rat. Under similar conditions, reserpine 5 mg/kg markedly increased excretion of this metabolite of serotonin(15). Thus, it appears unlikely that benactyzine affects 5-hydroxytryptamine metabolism. *Anticholinergic action on blood pressure.* Cats anesthetized with chloralose 60 mg/kg dissolved in 25% aqueous urethane were utilized in these experiments. After uniform blood pressure responses to acetylcholine 2 γ/kg and stimulation of the peripheral vagal stump (0.5 v. for 15 seconds) were obtained, benactyzine was injected and the tests repeated. Benactyzine 1 mg/kg reduced the depressor effect of both vagal stimulation and acetylcholine by about 30% while 3 mg/kg reduced the effects of both by more than 70%. *Potentiation of epinephrine.* The pressor effect of epinephrine in chloralosed cats was markedly greater after administration of benactyzine.

TABLE II. Concentrations of Benactyzine and Other Agents in γ/ml Required to Completely Antagonize Effects of Acetylcholine, 5-Hydroxytryptamine and Histamine on Smooth Muscle.

	Acetylcholine	5-Hydroxytryptamine	Histamine
Benactyzine	.05	1.0	5.0
Chlorpromazine	1.0	.5	.5
Reserpine	4.0	4.0	2.5
Meprobamate	10.0	10.0	100
Diphenhydramine	—	—	.25

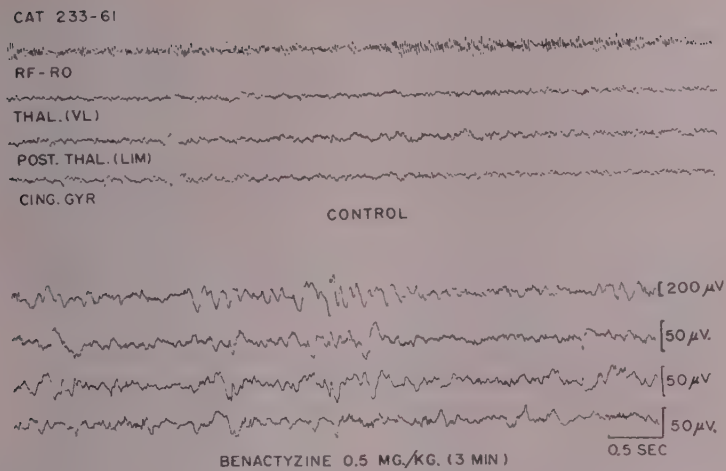

FIG. 1. Effect of benactyzine on electrical activity of brain. Recordings are from right motor to occipital cortex (RF-RO), nucleus ventralis lateralis of thalamus (VL), posterior border of thalamus, in nucleus limitans (LIM), and posterior cingulate gyrus (CING. GYR.).

Thus, benactyzine 1 mg/kg almost doubled the response previously obtained from epinephrine 5 γ/kg. Benactyzine 24 mg/kg in mice reduced the LD_{50} of epinephrine from 7.0 ± 1.3 mg/kg to 5.0 ± 0.3 mg/kg.

Effect on spinal reflexes. The knee jerk and flexor reflex in chloralosed cats showed no characteristic changes after benactyzine 3 mg/kg. After doses of 10 mg/kg both reflexes disappeared and respiration stopped. *Effect on brain waves.* In curarized cat preparations, benactyzine 1 mg/kg broke up the regular fast activity of the cortex (40/sec.). In its place, irregular slower outbursts with a predominant frequency of 8 to 15/sec. and higher than usual amplitude became apparent. A similar slowing and increase in amplitude was also noted in recordings from subcortical areas (Fig. 1). The drug also blocked EEG arousal from sensory or thalamic stimulation. Recruiting responses evoked in the cortex by thalamic stimulation were not affected. The records obtained were indistinguishable from those obtained after suitable doses of atropine under similar conditions.

Discussion. Benactyzine is closely related in chemical structure to several drugs that have been used in therapeutics for many years. The spasmolytic adiphenine (Trasentine) differs from benactyzine only in lacking the hydroxyl group in the benzilic acid portion of the molecule. Benactyzine is also similar to the benzilic ester of choline and the cycloplegic Lachesine(6) which differs from benactyzine only in that the nitrogen is quaternary. Benactyzine differs from other psychotherapeutic agents such as chlorpromazine, reserpine and meprobamate not only in chemical structure but also in pharmacological properties. The only action that these 4 psychotherapeutic agents have in common is the potentiating effect on hexobarbital anesthesia. This action, however, is not specific as it is shared by many other drugs, notably the antihistaminics. Benactyzine does not share with meprobamate the taming effect on monkeys, nor does it produce insulation from the environment and catatonia as do chlorpromazine and reserpine in this species. Its effects on spontaneous electrical potentials of the brain are like those of atropine. The ability of benactyzine to normalize stress-induced behavior in animals is of great interest but is not specific since similar effects can also be produced by alcohol(7). However, chlorpromazine appears to be ineffective when evaluated by this technic(7). The frequent occurrence of deaths in benactyzine-treated animals after normally non-lethal electroconvul-

sive shocks appears to make it inadvisable to give electroconvulsive treatment to patients taking benactyzine.

Summary. Benactyzine (β-diethylaminoethyl benzilate hydrochloride) an antispasmodic that has been used in the treatment of psychoneuroses causes hyperexcitability and clonic convulsions in animals. A taming effect was not observed. Hexobarbital anesthesia was markedly prolonged, the effects of acetylcholine on the isolated gut and blood pressure diminished and the action of epinephrine on blood pressure enhanced by this compound. Spinal reflexes were not affected and cortical and subcortical electrical activity was slowed. Mice receiving benactyzine often died during electroshock seizures which were tolerated by normal animals.

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The Development *in vitro* of an Avirulent, Immunogenic Variant of *Mycobacterium tuberculosis* var. *hominis*.* (22546)

ANNE S. YOUMANS AND GUY P. YOUMANS.
Department of Bacteriology, Northwestern University Medical School, Chicago, Ill.

Youmans and Youmans(1) reported that the virulent H37Rv strain of *Mycobacterium tuberculosis* var. *hominis* would multiply in a modified synthetic Proskauer and Beck medium from which the nitrogen source, asparagin, had been omitted, at a rate similar to that obtained in the complete medium. During the course of subsequent investigations, the H37Rv strain was subcultured serially once a week for approximately 6 years onto the surface of the Proskauer and Beck medium from which the nitrogen source had been omitted. It was found that the virulence of these cultures, when tested at intervals in mice and in guinea pigs, had decreased markedly. This variant of the parent H37Rv strain has been designated H37RaN, which stands for strain H37, rough, avirulent, nitrogen.

This study is concerned with a) the virulence of H37RaN as compared with the parent H37Rv, b) metabolic differences between the 2 strains, and c) the immunogenic activity of H37RaN as compared with the well known immunogenic mycobacterial strains, H37Ra and BCG.

Methods. The strains of mycobacteria employed were the 3 human strains, H37RaN, H37Rv, H37Ra, and the one bovine strain, BCG. The H37Rv and H37Ra strains were maintained as pellicle cultures on the modified synthetic Proskauer and Beck (P & B)

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medium(2) by weekly subculture. The variant, H37RaN, was grown in a similar fashion on the P & B medium except that the nitrogen source, asparagin, was omitted. Because of the slight filmy growth on this medium, it was necessary to make one subculture onto the complete P & B medium in order to have sufficient cells to make a suspension for experimental work. Cells of the BCG strain were kindly supplied by Dr. Sol Rosenthal of the Tice Clinic of Chicago at the time of each experiment.

Quantitative growth studies were done by employing the small inocula technic(3). This method consisted of inoculating a set of culture tubes with each of several inocula which varied in size from 10^{-1} mg through 10^{-6} mg, wet weight. The time in days when growth first occurred for each inoculum was recorded, and this figure was plotted against the logarithm of the inoculum size. From the straight line so obtained the rate of growth and generation time of the organisms were determined.

The basal medium employed was the modified P & B medium from which the carbon source, glycerol, was omitted. Dilutions of the carbon substrates related to the tricarboxylic acid cycle were made with the basal medium. The media were sterilized by sintered glass filtration and tubed aseptically. After inoculation, the cultures were incubated at 37°C and examined daily for the presence of growth. At least 2 tubes of each medium were used for each inoculum and all experiments were repeated 2 to 4 times.

In the animal experiments 3 procedures were used: a) The virulence of the H37RaN and the H37Rv strains was tested by injecting intravenously groups of 10 to 20 Strong A mice, 18-20 g in weight, with 1.0, 2.0 or 4.0 mg, wet weight, of a standardized ground suspension prepared from mature pellicles (3) grown on the complete P & B medium. The time when each mouse died and the autopsy findings were recorded. Following the method of Litchfield(4) the time of death, in days, was plotted against the cumulative percent mortality on logarithmic probability paper and from the straight line so obtained, the median survival time (ST_{50}) was determined, and the 95% confidence limits were calculated.

b) The virulence of H37RaN was tested in three 300 g guinea pigs by injecting 0.1 mg, wet weight, of the standardized ground suspension intraperitoneally. At the end of 4 months, the guinea pigs were killed, autopsied, and the organs examined macroscopically for tuberculous lesions.

c) The immunogenic activity of H37RaN, BCG and H37Ra was examined in mice by injecting intraperitoneally 1.0 mg, wet weight of standardized ground suspensions of live and heat killed cells. The heat killed cells were autoclaved at 126°C for 15 minutes. The mice were challenged 4 weeks later by infecting with 1.0 mg of H37Rv intravenously. In the evaluation of the results of these experiments, a different procedure was used. Immune mice die over a prolonged period after challenge and, consequently, the normal distribution curve on which the median survival time calculation is based is not obtained. Therefore, the percent of mice which lived longer than 29 days was used as an index of the degree of immunity, since approximately 95% or more of the controls die before this time.

Results. Morphology and cultural: H37RaN grew as a slight, filmy pellicle on the surface of the nitrogen-free P & B medium and resembled macroscopically the very young pellicle cultures of the virulent parent H37Rv strain. Cording was noted, and microscopically, the organisms were acid-fast and in all respects similar in appearance to the organisms of the parent H37Rv strain. On the complete P & B medium the H37RaN strain again resembled the growth of the H37Rv strain. The pellicle growth was luxuriant and did not contain "rough" patches. It did not resemble the heavy waxy pellicle growth produced by the avirulent H37Ra strain.

Virulence. The virulence of H37RaN was tested first in mice after one year of continual subculturing on the nitrogen-free P & B medium. The median survival time of the mice infected intravenously with 1.0 mg of H37RaN was 22.5 days, and the median survival time of the control mice infected with

TABLE I. Effect of Compounds Related to the Tricarboxylic Acid Cycle on Growth of Strains H37RaN and H37Rv of *M. tuberculosis* var. *hominis*.

Substrate	Concentration of substrate in %								
	1.0		0.25		0.06		0.016		0
Strains:	aN*	Rv†	aN	Rv	aN	Rv	aN	Rv	
Glycerol	26.4‡	21.7	10^{-3}§	21.7	10^{-3}	24.0	10^{-3}	24.0	
Lactic acid	10^{-2}	0	10^{-4}	22.7	10^{-2}	22.7	10^{-2}	22.7	
Pyruvic "	10^{-3}	0	24.0	20.5	10^{-2}	20.5		21.7	
Acetic "	0	0	10^{-2}	10^{-2}	10^{-4}	25.2	10^{-3}	25.2	
Citric "	0	0	0	0	0	0	0	0	
Oxalosuccinic acid			10^{-1}	0	28.8	10^{-2}	10^{-1}	25.2	
α-Ketoglutaric "	0	21.7	0	21.7	0	21.7	0	21.7	
Succinic acid	0	0	0	0	0	0	0	0	
Fumaric "	0	0	0	0	0	0	0	0	
Malic "	0	0	0	0	0	0	0	0	
Oxaloacetic acid		0	10^{-1}	21.7	10^{-1}	21.7	0	21.7	
DL-Alanine	0	0	0	0	0	0	0	0	
Glutamic acid	0	0	0	0	0	0	0	0	
Aspartic "	0	0	0	0	0	0	0	0	
None									0

* Strain H37RaN. † Strain H37Rv. ‡ Generation time in hr. § Smallest inoculum (in mg) which grew.

1.0 mg of the parent H37Rv strain was 14.5 days. The virulence of this strain was tested again after 3 years of continual subculturing on the nitrogen-free medium. The mice, which were infected with 1.0 mg of these organisms intravenously, were sacrificed 4 months after injection, and on autopsy revealed no macroscopic tuberculous lesions. Further experiments were done in which the amount of H37RaN injected intravenously into the mice was increased to 2.0 and to 4.0 mg. These mice were killed after 4 months and on autopsy no gross tuberculous lesions were seen. In comparison to these findings, mice infected with 2.0 mg and 4.0 mg of the parent virulent H37Rv strain had median survival times of 9.5 and 3.5 days, respectively. In the following 3 years, this strain has been subcultured on the same nitrogen-free medium, and the virulence has been tested several times in mice employing a 4.0 mg inoculum. None of the mice showed any evidence of tuberculosis, even those sacrificed as long as 8 months after injection. The virulence of this organism was tested in guinea pigs after the 3 year period and also appeared to be non-virulent for these animals. The animals were sacrificed 16 weeks after injection, and on autopsy found to have no gross tuberculous lesions. The H37RaN strain was subcultured serially at weekly intervals on the complete P & B medium to determine whether virulence would be restored by the addition of the nitrogen source, asparagin. The last such subculture to be tested for virulence in mice was the 36th. Four of the 10 mice injected with 3.0 mg died between the 2nd and 7th months of tuberculosis and the remainder were sacrificed 7 months after infection. Upon autopsy, the lungs of these mice were grossly tuberculous with about 25-50% of the lung tissue involved.

Metabolic studies. The results of these studies are shown in Table I. If growth occurred with several dilutions of the inocula, thus permitting the rate of growth to be determined, the generation time in hours is recorded; otherwise, either the smallest inoculum which grew, or no growth, is recorded. The results with the H37Rv strain given in the table were the same as those reported previously(5). A generation time for H37RaN could be determined for only one concentration of glycerol, pyruvic and oxalosuccinic acids. The growth of only the larger inocula of H37RaN was supported by lactic, acetic, oxaloacetic acids. Alpha-ketoglutaric acid did not support the growth of any inoculum. The parent H37Rv strain grew well in several concentrations of each of these substrates. No growth occurred with either strain in the presence of citric, isocitric, *cis*-aconitic, succinic, fumaric, malic, as-

TABLE II. Response of Immunized Mice to Infection with *Mycobacterium tuberculosis* var. *hominis* (H37Rv).

Vaccine inj. (1 mg)	No. of mice*	% lived >29 days
H37RaN	35	82.9
H37RaN (heat killed)	20	50.0
H37Ra	40	77.5
H37Ra (" ")	39	35.9
H37Rv (" ")	38	34.2
BCG	68	82.3
BCG (" ")	71	31.0

Less than 10% of the control mice lived longer than 29 days.

* Mice which died before challenge are not included.

partic, glutamic acids and alanine.

Immunogenic studies. The results of these experiments are found in Table II. Live cells of H37RaN, H37Ra, and BCG strains immunize to a similar degree, as do the killed cells of H37RaN, H37Ra, BCG and H37Rv; however, killing the cells with heat significantly reduced the immunogenic activity of the strains. The values given in the table which indicate the number of mice which lived longer than 29 days are somewhat high. In other experiments in this laboratory, these strains have produced a lesser degree of immunity.

Summary. A variant of the H37Rv strain of *Mycobacterium tuberculosis* var. *hominis* which appears to be avirulent for mice and guinea pigs, was developed *in vitro* by continual subculture over 3 years on a nitrogen-free synthetic medium. This new strain, designated H37RaN, resembled the parent H37Rv strain both macroscopically and microscopically, but differed metabolically from the parent strain in its reduced ability to utilize for growth carbon compounds related to the tricarboxylic acid cycle. This strain immunized mice as well as the frequently used immunogenic strains, H37Ra and BCG.

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Notes on Impeded Estrogens. (22547)

RICHARD A. EDGREN. (Introduced by F. J. Saunders.)
Division of Biological Research, G. D. Searle and Co., Chicago, Ill.

Huggins and Jensen(1) have recently discussed a category of estrogenic substances which they have called "impeded estrogens," and defined as compounds producing dose-response curves with shallow slopes when assayed on uterine growth. The prototype of this group of substances is estriol. All of the impeded estrogens studied by Huggins and Jensen possessed either a ketone group at position 6 or a hydroxyl group at 16 leading them to conclude that an oxygenated group at 6 or 16 is necessary for impeded status. They also stated that 16-oxoestrone (16-ketoestrone) did not cause an impeded uterine growth response. Certain of these substances have been shown to have the property of inhibiting the uterine stimulating action of estrone when administered simultaneously with estrone(1,2). Observations made in this laboratory confirmed Huggins and Jensen only in part and suggested that extensions of their conclusions are warranted.

Materials and methods. The assay procedure of Rubin *et al.*(3) has been followed. Mice 23-25 days of age were used; the total dose of test material was administered in 3 divided doses in 0.1 ml corn oil each. The injections were given daily for 3 days and

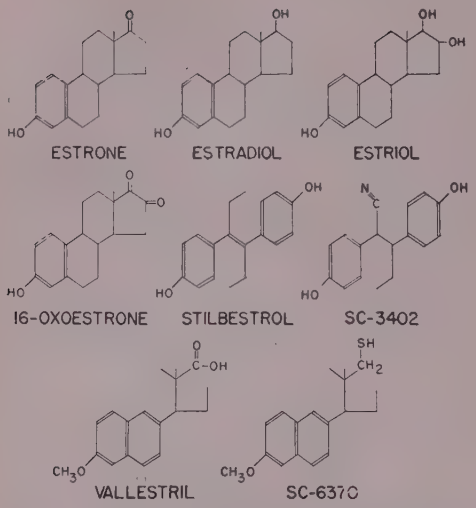

FIG. 1. Structural formulae of estrogenic substances showing widely varying dose-response curve slopes.

autopsy was performed 24 hours after the final injection when the animals were 26-28 days old.

At autopsy the uteri were removed, cleaned of adherent tissues, scored and blotted to express contained fluid, and weighed wet on a Roller-Smith torsion balance. Body weights were also obtained, but were used only as a rough index of age and health of the mice. The mean uterine weights of groups of animals (8-10 per group) at various dose levels were fitted to log-dose-response curves over the linear portions of the regressions using the method of least squares(4,5).

The compounds studied included 3 natural steroids: estrone, estriol, and estradiol 17β; 2 commercial synthetic substances: diethylstilbestrol and 2,2-dimethyl-3-(6-methoxy-2-naphthyl)-pentanoic acid (Vallestril); a steroid analog: the higher melting racemic 2, 3-bis (p-hydroxyphenyl)-valeronitrile (designated SC-3402)(6); a Vallestril derivative: 2,2-dimethyl-3-(6-methoxy-2-naphthyl)-pentanethiol (designated SC-6370; and 16-oxoestrone. Structural formulae for these compounds are presented in Fig. 1.*

*SC-3402, SC-6370 and 16-oxoestrone were synthesized by Drs. Kurt Rorig, Paul Sollman and David Tyner, respectively, of the Chemical Research Division, G. D. Searle and Co.

Results. Regression coefficients for the compounds studied are shown with their standard errors in Table I. These compounds appear to fall into 3 categories, irrespective of potency: Those with extremely steep slopes (estrone, Vallestril), those with slopes of intermediate values (estradiol, diethylstilbestrol), and the impeded estrogens (estriol, SC-3402 and 16-oxoestrone). SC-6370 had a slope which fell between the latter 2 groups and did not differ significantly from either.

Discussion. Under the condition of our test, estrogenic substances appeared to fall into 3 categories based upon slope of the dose-response curve in contrast to the 2 classes obtained by Huggins and Jensen(1). It seems not improbable that more extensive study will uncover additional classes of regression coefficients or perhaps even a spectrum of slopes, especially in view of the fact that SC-6370 had a slope which fell midway between the impeded and intermediate groups. These 3 groups are each made up of both natural estrogens and synthetic substances which can be considered steroid analogs. The compounds with shallow slopes which have been discussed here cannot be categorized on the basis of 6 or 16 oxygenation as was true of structures studied by Huggins and Jensen. The structure-activity relations of the impeded estrogens would appear somewhat more complex than was apparent previously. The impeded nature of 16-oxoestrone also differs from the statements of Huggins and Jensen. The discrepancies between our findings and theirs may result in part from species differences and from the fact that they employed hypophysectomized animals.

Summary. Using the uteri of immature

TABLE I. Regression Coefficients of Certain Estrogenic Compounds. N = No. of groups of 8-10 mice used in computation of slopes.

Compound	N	Regression coefficient
Estrone	23	43.3 ± 3.64
Vallestril	29	41.6 ± 4.13
Diethylstilbestrol	15	29.6 ± 5.98
Estradiol 17β	12	25.5 ± 5.79
SC-6370	9	18.2 ± 3.43
16-oxoestrone	8	9.4 ± 4.82
Estriol	7	9.2 ± .93
SC-3402	6	7.4 ± 1.76

mice as a test object, the slopes of the dose-response curves for several estrogens appear to fall into 3 or more categories. In addition to estriol and 16-oxoestrone, a substance related structurally to stilbestrol showed a shallow slope, and a Vallestril derivative showed a slope steeper than the impeded estrogens, but not as steep as those of certain standard substances.

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Determination of Respiratory LD_{50} from Number of Primary Lesions as Illustrated by Melioidosis.* (22548)

Arthur M. Dannenberg, Jr.† and Eva M. Scott.
(Introduced by Albert P. Krueger.)
From the Naval Medical Research Unit No. 1 and the Naval Biological Laboratory, Department of Bacteriology, University of California, Berkeley.

Lurie *et al.*(1) have used the number of primary pulmonary foci as a quantitative index of resistance to experimental tuberculosis as well as an index of the virulence of the infecting bacteria. In this report it is shown that in melioidosis, where one primary pulmonary lesion is fatal to mice and hamsters, it is possible to use this lesion-count method to determine the respiratory LD_{50}. This method shows good agreement with the usual titration method and has certain advantages over the latter.

Materials and methods. Mice, Namru strain(2), and golden hamsters were exposed to aerosols of virulent *Malleomyces pseudomallei*, strain 103-67, using a Wells-type atomizer and the method described by Leif and Krueger(3). After the animals died, the lungs were removed and inflated by injecting fixative (FU 48 Technicon) intratracheally. The primary pulmonary lesions were yellow and easily distinguishable from the tan to brown background of the fixed lung parenchyma (Fig. 1).

The number of organisms in the inhaled dose was calculated from colony counts of aerosol aliquots cultured on 4% glycerine beef extract agar. The number of pulmonary lesions was determined either by dissecting the lung if the lesion count was 25 or less, or using the graph shown in Fig. 2 if the lesion count was above 25.

Determination of Respiratory LD_{50} from total number of lung lesions. A single lung lesion in experimental melioidosis in mice and hamsters is always fatal since only progressing pulmonary lesions have been found. Almost without exception, such progressing primary lesions do not occur elsewhere in the body following exposure to aerosols of the organism. It is therefore possible to determine the number of organisms necessary to generate one pulmonary melioidotic lesion, or the "ratio," by dividing the inhaled dose by the number of lung lesions. This "ratio" is an expression of virulence and the respiratory LD_{50} is equal to the "ratio" multiplied by

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† LCDR, MC, USNR.

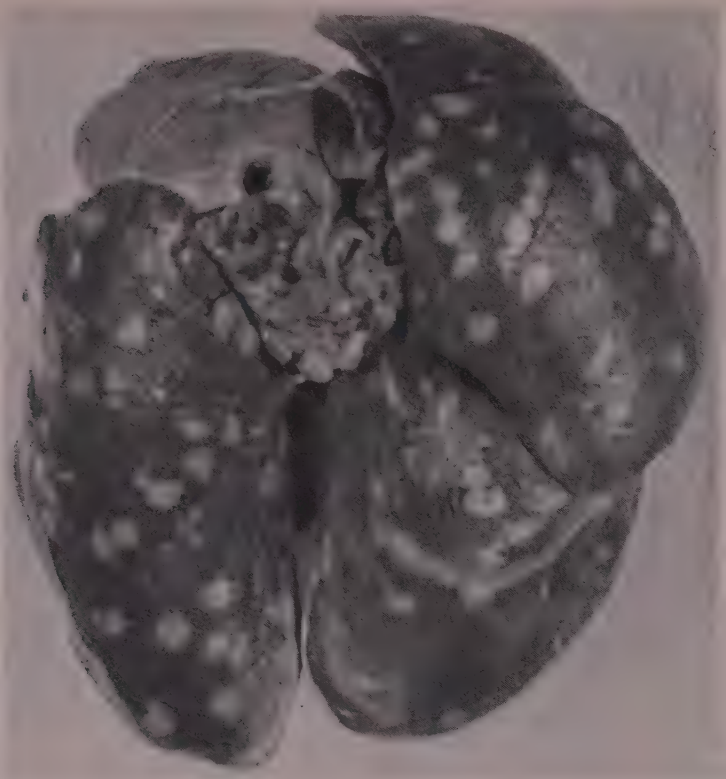

FIG. 1. Lung of a mouse dying 5 days after inhalation of 1.1×10^4 virulent *Malleomyces pseudomallei*, strain 103-67. This was fixed by intratracheal inj. of FU 48 (Technicon).

0.7.‡ This lesion-count method of calculating the respiratory LD_{50}, which seems correct theoretically, is also supported by experimental data. Table I lists mean LD_{50} values obtained by this method as well as by the titration method(4). The 95% confidence intervals for these mean LD_{50} values were determined by using a modification of the binomial distribution(5). The confidence intervals for the lesion-count method were found to overlap those for the titration method. It is probable that an even greater overlap exists than that illustrated, because the total lesion counts which were derived from Fig. 2 were assumed to have the same precision as the direct lesion counts.

Estimation of total number of pulmonary lesions when they are too numerous to count. The accurate counting of about 25 lesions per lung in mice and hamsters is not difficult, but as the number of lesions increases, inaccuracies occur. Reasonable estimates of the latter may be made by assuming that (1) each lesion started as a completely isolated focus of infection regardless of the dose, and (2) the ratio of the number of inhaled organisms to total lesions was the same for high as for low dosages. This is predicated on the observation that the number of alveoli in the lung far exceed the number of bacteria inhaled. Based on actual counts on histological sections, it was estimated that there were approximately 40 million alveoli in mice and 160 million alveoli in hamsters. Maximal doses in experimental melioidosis never exceeded one million organisms. Since approxi-

‡ If all the animals showing lesions had but a single lesion, the average lesion count in a group showing 50% mortality would equal 0.5. Since some animals have more than one lesion, the average is raised to 0.7 lesion, actually 0.693 lesion, because of Poisson's distribution.

TABLE I. Comparison of Respiratory LD_{50} Values Obtained by Lesion Count and Titration Methods in Mice and Hamsters Infected with *M. pseudomallei*, Strain 103-67.*

No. of animals	Dose inhaled (organisms)	Avg surface lesion count	Total lesions†	Ratio‡	Organisms per LD_{50}: Lesion count method, Ratio × 0.7	Lesion count method, Geometric mean§	Titration method, geometric mean§
			Mice				
8	4.2×10^0		.25	17	12		
7	1.4×10^1		1.0	14	10		
8	1.3×10^2		7.3	18	12		
10	3.3×10^2	33	44	75	53	17 (10–28)	9 (5–17)
10	3.8×10^1		1.6	24	17		
10	2.5×10^2		5.0	50	35		
10	1.3×10^3		21.0	62	43		
10	1.3×10^4	85	325	40	28		
10	2.3×10^5	369	6900	33	23	28 (18–43)	16 (8–32)
7	3.6×10^1		1.0	36	25		
8	3.1×10^2		8.9	35	25		
8	1.8×10^3	34	48	38	27		
4	1.0×10^4	138	880	11	8		
6	1.3×10^5	363	6500	20	14	18 (11–31)	25 (12–59)
			Hamsters				
7	9.3×10^0		0	—	—		
8	4.0×10^1		3.1	12	9		
6	2.9×10^2		7.4	39	27		
6	6.6×10^2	45	85	78	55		
6	8.6×10^2	59	150	57	40	27 (15–42)	22 (9–40)
9	3.4×10^1		1.2	28	20		
10	2.7×10^2		2.2	123	86		
8	2.6×10^4	142	960	27	19		
4	6.6×10^4	186	1700	39	27		
6	7.3×10^4	176	1500	49	34	31 (19–51)	11 (6–20)
8	2.4×10^1		1.3	18	13		
6	9.6×10^1		2.0	48	34		
6	2.9×10^2		4.8	60	42		
7	1.7×10^3	34	48	35	25		
7	1.5×10^4	68	200	75	53		
7	1.1×10^5	509	10000	11	8	24 (15–38)	13 (6–29)

* Incomplete data from experiments employing other strains of *M. pseudomallei* or vaccinated, rather than normal, mice and hamsters also supported the principles presented in this report.

† Counts above 25 estimated by Fig. 2.

‡ Variation observed in ratios is probably inherent in the exposure apparatus rather than in sampling or lesion-counting technics.

§ 95% confidence interval in parentheses.

mately 32§ inhaled organisms were required to produce one lesion in mice (with strain 103-67), the total number of lesions was calculated by dividing the inhaled dose by 32 (Table II). Plotting the total lesions calculated in this way against the surface lesion count in mice resulted in the curve shown in Fig. 2 which in turn serves to estimate the total lesions in mice from any surface lesion count. Fig. 2 also shows a similar curve for hamsters. After an estimate of the total number of lesions is obtained, the ratios may be computed by dividing the calculated inhaled dose of organisms by this estimate. From such a ratio an estimate of the organisms per LD_{50} is determined by multiplying by 0.7.

Discussion. The advantages of the lesion-count method as compared to the titration method for determining the respiratory LD_{50} are that (1) the LD_{50} can be obtained with the former even if all the animals die or are killed; (2) the former is probably more accurate; and (3) fewer animals and exposures

§ 32 is the geometric mean of "ratios" obtained from average lesion counts of 25 or less.

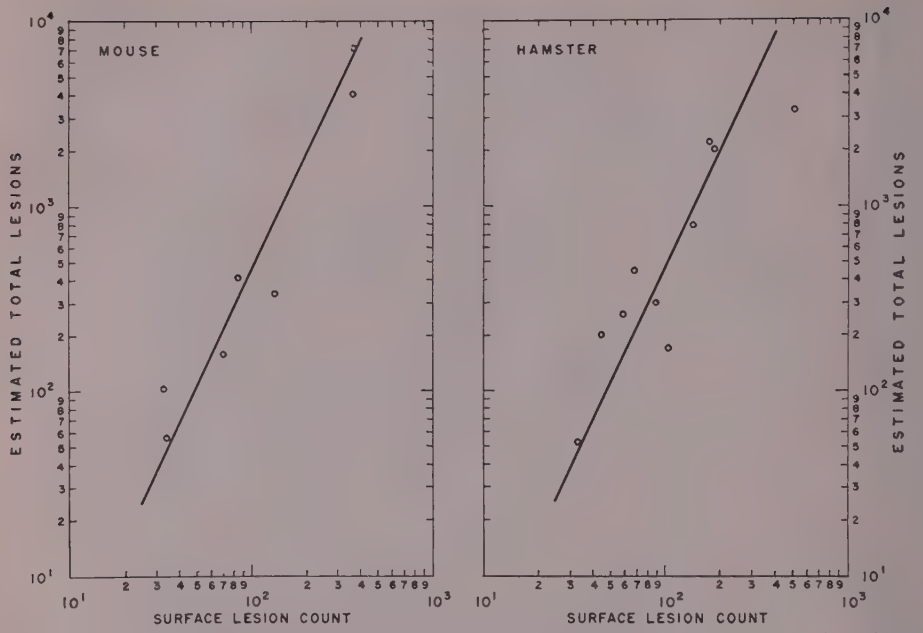

FIG. 2. Estimated total pulmonary lesions in mice and hamsters from number of surface lesions. (See Table II for derivation.)

are required. This latter point can mean considerable financial saving when monkeys are employed in such determinations. It should be mentioned that with the lesion-count method an LD_{50} can be computed from a single cloud exposure and it should therefore be possible to compare many more experimental variables simultaneously. The lesion-count method may also be used in conjunction with the usual titration method. The graphs relating the total number of pulmonary lesions in mice and hamsters to the surface lesion count are only approximations. A certain amount of error would be introduced should the pulmonary lesions be the same in number but smaller in size than those used to construct these graphs since fewer lesions would reach the surface of the lung. Also the converse is true. Nevertheless, these graphs should find application in studies with this and other organisms that produce discrete pulmonary lesions because they yield information from experiments that might otherwise be useless.

Summary. 1. A method is described whereby the respiratory LD_{50} can be obtained from the dose of organisms inhaled and from counts of primary pulmonary lesions, provided a single pulmonary lesion is fatal and progressing primary lesions do not occur elsewhere. This

TABLE II. Estimation of Total Pulmonary Lesions in Mice and Hamsters when Number of Surface Lesions Is Known. (Data for Fig. 2.)

No. of animals in exp.	Dose inhaled (organisms)	Estimated total No. of lesions*	Avg surface lesion count
	Mice		
8	1.8×10^3	56	34
10	3.3×10^3	103	33
15	5.1×10^3	159	70
4	1.1×10^4	340	138
10	1.3×10^4	410	85
6	1.3×10^5	4100	363
10	2.3×10^5	7200	369
	Hamsters		
7	1.7×10^3	52	34
5	5.6×10^3	170	104
6	6.6×10^3	200	45
6	8.6×10^3	260	59
5	1.0×10^4	300	90
7	1.5×10^4	450	68
8	2.6×10^4	790	142
4	6.6×10^4	2000	186
6	7.3×10^4	2200	176
7	1.1×10^5	3300	509

* Dose/32 for mice and dose/33 for hamsters.

method was tested and confirmed in experimental melioidosis in mice and hamsters, and its advantages over the usual titration method were discussed. 2. The number of *M. pseudomallei* necessary to generate one lesion, called the "ratio," is obtained by dividing the inhaled dose by the total number of primary pulmonary foci. This "ratio" multiplied by 0.7 equals the respiratory LD_{50}. Data and graphs are presented to estimate the total number of primary pulmonary lesions when they are so numerous that only the lesions on the surface of the lung can be counted. Estimates of the 95% confidence interval of the respiratory LD_{50} obtained by this lesion-count method overlapped those obtained by the titration method.

We wish to acknowledge the statistical advice of Mr. Leonard Goldberg, the technical assistance of Roy Forcier, HMC, and William Eisenman, HM3, as well as the aid of Dr. Clara Nigg in the preparation of the manuscript.

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A Chick Embryo-Derived Complement-Fixing Antigen for Western Equine Encephalomyelitis. (22549)

EDWIN H. LENNETTE, ANNA WIENER, BEVERLY JEAN NEFF, AND MARJORIE N. HOFFMAN.

Viral and Rickettsial Disease Laboratory, California State Department of Public Health, Berkeley.

Complement-fixing antigens for the diagnosis of certain neurotropic viral infections, for example, Western equine encephalomyelitis, St. Louis encephalitis, Japanese B encephalitis, are in general derived from infected rodent (usually mouse) brain tissue. Howitt(1) used ether-extracted mouse brain as an antigen for the detection of antibodies to the Western equine and St. Louis encephalitis viruses but found that the margin between specific and nonspecific reactions was narrow. Casals and Palacios(2), also using mouse brain as an antigen source, were able to remove some of the nonspecifically reacting substance by means of repeated freezing and thawing. Havens *et al.*(3) used high-speed centrifugation to remove nonspecifically reacting substances from mouse and hamster brain antigens.

In the case of the Western equine encephalomyelitis virus, complement-fixing antigens have also been prepared from homogenized chick embryos. Mohler(4), who used formalinized preparations, found them to be highly anticomplementary and frequently nonspecific. Brown(5), however, found that homogenized embryo suspensions, either uninactivated or inactivated by ultraviolet irradiation, gave specific fixation with guinea pig hyperimmune sera. One of the difficulties with WEE (and EEE) mouse brain antigens has been the low antibody titers obtained with human sera (WEE(6,7)) and horse sera (EEE and WEE(8)), and also the occurrence of nonspecific results(6). DeBoer and Cox(6) found that antigens prepared from homogenized chick embryos or from mouse brains treated by repeated freezing and thawing reacted with syphilitic sera and that this non-specific reactivity could be removed by benzene extraction. España and Hammon(9) modified the method in order to obtain more sensitive antigens. Preparation of mouse brain antigen by the method of DeBoer and Cox requires approximately 4 days(9), and by the method of España and Hammon, approximately 36 hours(9). A simpler and less expensive means of preparing antigens is de-

sirable, and the present communication describes a WEE virus antigen prepared from the embryonated hens' egg.

Preparation of WEE egg antigen. Eleven-day-old embryonated hens' eggs are inoculated into the allantoic cavity with 0.1 ml of egg-passaged WEE virus in 10% normal rabbit serum broth. The virus suspension is appropriately diluted so that approximately 50% of the embryos will be moribund within 24 to 30 hours. The A-42 strain of WEE virus, which was isolated in this laboratory in 1946 from the brain of a horse and which has been passaged only in the chick embryo since its original isolation in the egg, is used. As soon as moribund embryos are detected, they are placed in the refrigerator, and when approximately 50% of the embryos are moribund, the remainder of the eggs is placed in the refrigerator and held overnight at 4°-6°C. The next day, the allantoic and amniotic fluids are harvested and pooled, and the allantoic and amniotic membranes are removed and also pooled. The membranes are made into a 50% suspension in the fluids either by grinding in a mortar or by a 2- to 3-minute cycle in a blendor. The suspension is spun at 10,000 rpm in the angle rotor of a Spinco centrifuge for one hour. The clear supernatant fluid is removed and merthiolate is added to give a final concentration of 1:10,000. This constitutes the final antigen, which is distributed into ampoules of convenient size and stored on dry ice. The antigen yield from 48 embryonated eggs approximates 75 ml.

Detection of WEE complement-fixing antibody in human sera. Egg membrane-fluid antigens, prepared essentially as described above, have been used in this laboratory continuously since 1950. Up to 1953, the antigen suspension was centrifuged at 4500 rpm for one hour in an angle head at 4°C in a refrigerated International centrifuge; preparations spun at 10,000 rpm for one hour in a Spinco machine (refrigerated) have been used since then, as a much clearer antigen is produced, and the incidence of nonspecific reactions is also reduced. Complement fixation tests for the detection of WEE virus antibodies were performed according to the technic described several years ago(10), and latterly according to a slightly modified method recently described(11).

TABLE I. Results of Complement Fixation Tests with WEE Egg-Derived Antigen and Sera of Patients with Encephalitis.

Patient No.	Name	Days after onset	WEE antibody — CF titer	WEE antibody — Neutralization indices
1	H. Ca	24	<1:8	1.8
		45	1:16	1.7
2	G. Da.	6	<1:8	1.9
		13	1:16	1.9
3	G. Ag.	5	<1:8	2.6
		23	1:32	1.7
4	F. Ar.	5	<1:8	2.5
		17	1:32	3.0
5	J. Ba.	1	<1:8	1.6
		18	1:32	2.9
6	L. Ga.	12	<1:8	1.9
		26	1:32	2.1
7	C. An.	6	<1:8	2.1
		16	1:64	2.1
8	D. Br.	8	<1:8	3.1
		22	1:64	2.9
9	G. Cl.	2	<1:8	1.0
		14	1:64	2.1
10	E. LaS.	2	<1:8	1.9
		18	1:64	2.7
11	I. Br.	9	<1:8	1.8
		22	1:128	2.7
12	R. Ac.	4	<1:8	1.9
		23	1:128	>3.2
13	E. Cl.	4	<1:8	3.4
		19	1:128	4.0
14	M. Et.	10	<1:8	1.3
		19	1:128	2.4
15	M. Fr.	4	<1:8	1.3
		14	1:128	2.4
16	L. Ki.	2	<1:8	1.9
		12	1:128	2.0
17	J. Bo.	5	<1:8	2.6
		19	1:256	2.1
18	F. Fa.	6	<1:8	1.6
		13	1:512	2.2
19	I. Ce.	9	1:32	1.6
		24	1:512	2.9
20	M. Ch.	7	1:64	3.1
		21	1:256	3.3

Table I illustrates rises in complement-fixing antibody in 20 patients with encephalitis due to WEE virus infection. By the time the first blood specimen was obtained after onset of illness, neutralizing antibody had already attained maximal levels, *i.e.*, it was not possible to show a diagnostically significant rise in titer between acute and convalescent phase serum specimens. The neutralization

test employed was the usual one in which undiluted serum is tested against serial 10-fold dilutions of the virus by intracerebral inoculation of the mixtures into adult Swiss mice. Since a change in virus titer of one dilution step (10-fold or 1.0 log) is within the experimental error of this method, and if a rise of 1.5 logs in the neutralization indices is considered as the minimal significant change(12), then only in Patient 12 could a diagnosis be made by a significant rise in neutralizing antibody level. On the other hand, it was possible to show a rise in complement-fixing antibody in all 20 individuals. The patients listed in Table I represent only 20 of the more than 350 cases of Western equine encephalitis diagnosed in the 1952 outbreak in California(12) by means of complement fixation tests in which an egg membrane-fluid antigen was used.* In 18 of the 20 patients listed, the complement-fixing antibody titer of the acute phase specimen was less than 1:8. This was typical of the preponderance of the entire series of patients mentioned; of this group, only a very small number had antibody levels of 1:8 or greater in the acute-phase serum specimen.

The egg antigen described here is easily prepared and, equally important, preparations of good antigenic potency (one unit = 1:16 or 1:32 dilution) can be consistently prepared. In our hands, antigen prepared from infected mouse brain by Casals' modification(13) of the original freeze-thaw method of Casals and Palacios(2) while satisfactory, varies as to antigenic potency; occasional lots approaching the potency of the egg antigen are obtained, but in general the antigenic potency is lower. An occasional human serum will give some degree of nonspecific fixation with a control egg membrane antigen prepared in the same fashion as the specific antigen. In such cases, the sera are retested with mouse brain antigen prepared by the Casals method(13).

Summary. A complement-fixing antigen of the WEE virus is described. The antigen is prepared from the fluids and membranes of the infected chick embryo and has a high degree of sensitivity. Since complement-fixing antibody in human WEE virus infections is slower in appearing than the neutralizing antibody, diagnosis can usually be made by the complement fixation method whereas diagnostically significant rises in antibody frequently are not demonstrable by the neutralization test.

* The patients in this series were tested with a "4500 rpm" antigen. The "10,000 rpm" antigen subsequently adopted gives essentially similar results with respect to specific fixation, but is freer of nonspecifically reactive substances due to the higher gravitational field of force applied during centrifugation.

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Production of Beak and Skeletal Malformations of Chick Embryo by Semicarbazide. (22550)

Robert E. Neuman, Merle Maxwell and Thomas A. McCoy.*
(Introduced by S. H. Wender.)
Biomedical Division, Samuel Roberts Noble Foundation, Ardmore, Okla.

Aminoguanidine injected into the yolk of the embryonated egg produced a severe and characteristic inhibition of development of the chick embryo liver(1). The present paper reports the effect of the structurally related semicarbazide • HCl upon the development of the chick embryo. This compound produces a profound distortion of the beak and leg bones with only minor lesions of the liver.

Methods. The semicarbazide • HCl (SCH)† was dissoved in water and adjusted to pH 4.5-5.0. All solutions and water used for dilutions were sterilized using a Selas filter of 03 porosity. The embryonated White Leghorn eggs were incubated for 96 hours, and the solutions (0.4 ml) were injected into the yolk. After further incubation (usually for a total of 14 days), the embryos were removed for examination.

Results. The effect of SCH on mortality, skeletal structure and body weight of the embryo is recorded in Table I. Injections of SCH in amounts exceeding the 3 mg level resulted in 100% mortality. Most of the embryos died in the 3 mg treatment, but the percentage of survival increased sharply below this level. Irrespective of the levels tested, SCH had little effect on the body weight of the surviving embryos. Of particular interest was the skeletal malformation produced when SCH was administered at 2 and 3 mg/egg dosages. When injected at 4 days and harvested at 14 days, the lower beak of the embryo was shortened and distorted with an upward sharp bend, and the tibiotarsus was sharply bent (Fig. 1, 2). Other tissues appeared normal, although the liver occasionally revealed minute whitish areas at the borders of the lobes. Malformation occurred in milder form and with lowered incidence as the dosage levels were decreased.

The effect of different concentrations of SCH administered at embryonic ages from 0-16 days was then determined. Injections at incubation periods earlier than 4 days proved less effective, and administration of SCH at 7-10 days produced distortion and irregularities in that the legs frequently were twisted or flattened and beaks were crossed and/or twisted. The total effects of the 2-3 mg/egg dosage occurred to a decreasing degree as the time of injection approached 12 days. The 12-day embryos were practically normal. The most severe effect of SCH was obtained by injections at 6 days. At the 2.0-2.5 mg dosages, ¾ of the embryos had, in addition to the sharp bending of the tibiotarsus, another distinct bending of the tarsometatarsus. These effects are strikingly similar to that of hereditary chondrodystrophy in the fowl(2).

TABLE I. Effect of Semicarbazide • HCl on Mortality, Skeletal Structure and Body Weight of Chick Embryo.

Quantity inj. (mg/egg)	No. of eggs	Embryos survived	Wt at 14 days (mean ± avg dev.)
0	6	6*	10.09 ± .75
1.0	4	4†	9.70 ± 1.09
1.5	8	7‡	9.86 ± .24
2.0	8	7§	8.88 ± .83
2.5	8	6‖	8.46 ± .51
3.0	6	2¶	9.22 ± .98
4.0	4	0	—
5.0	4	0	—

* All normal.

† One embryo with normal beak but bend in tibiotarsus; other embryos apparently normal.

‡ One embryo with malformed beak but normal legs; other embryos with bent tibiotarsus and malformed lower beak.

§ All embryos with bent tibiotarsus, legs not folded back behind body; one embryo with normal beak, one embryo with slightly malformed beak.

‖ All embryos with acutely bent tibiotarsus, legs tend to fold behind back; beaks malformed.

¶ Legs of all embryos with acutely bent tibiotarsus and folded on back; beaks malformed.

* The authors gratefully acknowledge technical assistance of Mr. Eugene Conway and Miss Rosaria M. Giacino.

†Distillation Products Industries, Rochester, N. Y.

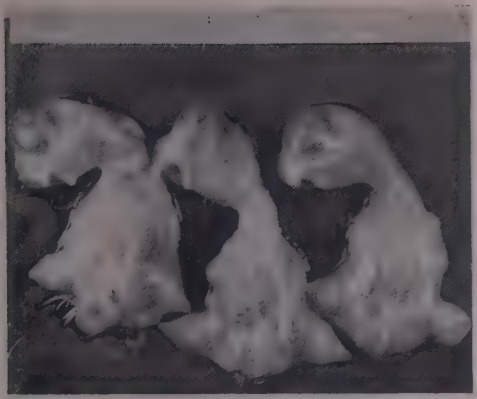

FIG. 1. Appearance of 14-day embryos treated with 2.5 mg semicarbazide • HCl at 4 days. Note tibiotarsus bent to such degree that remainder of leg is folded under embryo.

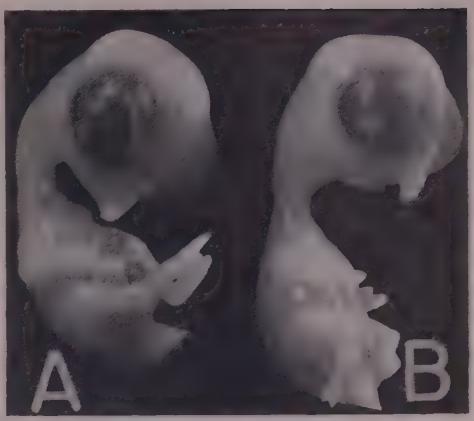

FIG. 3. Appearance of 8-day embryos. A—Control; B—Treated with 2 mg semicarbazide • HCl 48 hr previously.

Eggs were injected after 6 days incubation and embryos were examined at 24-hour intervals up to an age of 16 days. A gross malformation of the beak and legs was evident as early as 48 hours after injection (8-day-old embryo). This is shown in Fig. 3. The embryos were fixed and stained. In Fig. 4, sections of the bones from normal and treated 8-day-old embryos are compared and tibiotarsal and tarsometatarsal bends at increased magnification are shown. It is difficult to ascribe the bending to histological changes. A closely related compound, acetone semicarbazone, was toxic at the same level as SCH, and the typical SCH syndrome was produced at the 2.5 mg/egg level. If the semicarbazone dissociated in the egg, the results may have been due to SCH *per se*.

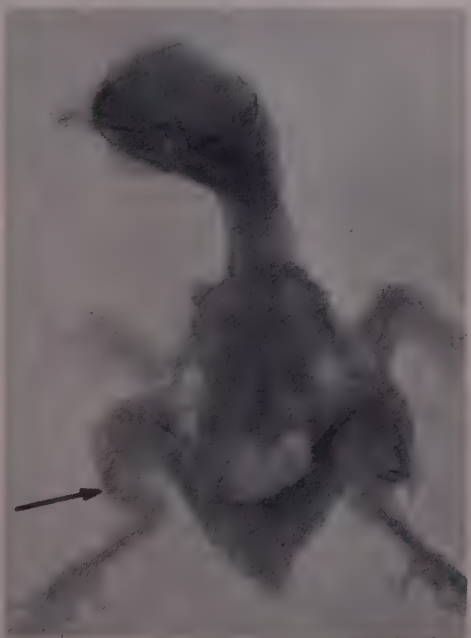

FIG. 2. X-ray photograph of 16-day embryo treated with 2.0 mg semicarbazide • HCl at 4 days.

When the carbonyl reacting portion of SCH was blocked as in 1-phenylsemicarbazide, the resulting structure was still capable of producing abnormalities. The 2.0-6.0 mg dose of 1-phenylsemicarbazide produced severe foreshortening of the legs, feet and wings; shortened beak; whitish areas of the borders of the liver: and moderate to extreme edema. The gizzards in half of the embryos examined were bloated with a clear viscous fluid. Thiosemicarbazide was much more toxic (L.D. 0.5 mg/egg) than SCH. At lower concentrations about 5% of the embryos displayed a bend in the tibiotarsus. p-Hydrazinobenzoic acid caused effects similar to those of SCH, but benzoic hydrazide did not produce abnormalities.

Discussion. SCH and hydroxylamine have been recognized as carbonyl reacting reagents. Zeller(3) used them to inhibit diamine oxidase and suggested that the oxidase contained ketone or aldehyde groups which reacted with diamines. Subsequently, SCH has been used

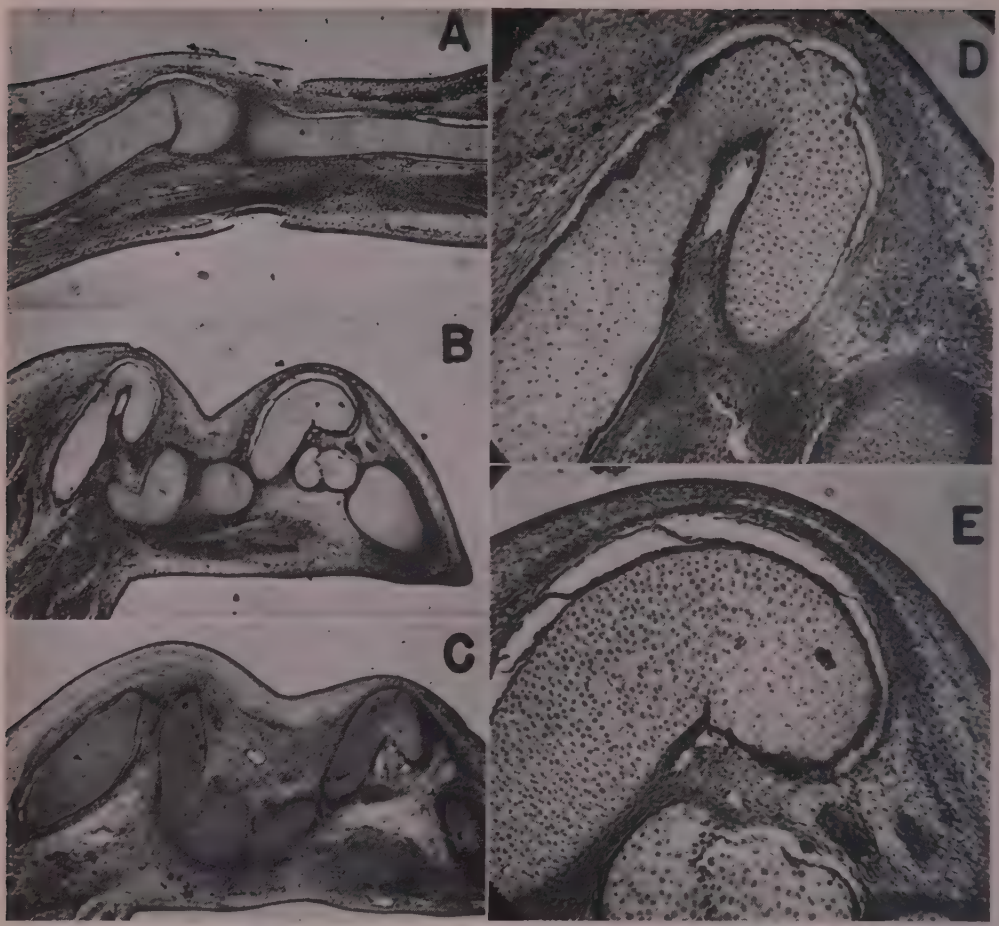

FIG. 4. Median sections of tibiotarsal and tarsometatarsal portions of leg bone of 8-day chick embryo when inj. at 6 days. A—Control, × 25. B and C—Typical sections from treated embryos (2 mg SCH/egg). Examination of serial sections revealed that tibiotarsus and tarsometatarsus were intact; × 25. D—Enlarged tibiotarsal section of B, × 100. E—Enlarged tarsometatarsal section of B, × 100.

to inhibit other enzymes such as, pancreatic lipase(4), the deaminating enzymes of histamine, isoamylamine and putracine(5), and spermine oxidase(6). In the spermine oxidase studies, Hirsch reported that SCH would specifically block the enzyme while certain amines and cyanides would bring about only partial inhibition. Clark *et al.*(7) reported the isolation of an enzyme from hog and guinea pig kidney which would specifically decarboxylate 5-hydroxytryptophan. Semicarbazide • HCl would almost completely inhibit this enzyme in concentrations of 10^{-3} M which suggested that the coenzyme involved may be pyridoxal phosphate. Addition of pyridoxal phosphate would not reverse the inhibition as it has been shown to do in the case of DOPA decarboxylase when inhibited by SCH. Shulman(8) reported that 0.26-0.34 M concentrations of SCH would reversibly inhibit the clotting of fibrinogen, indicating that the inhibition did not involve the destruction of the proteins involved. Sang and McDonald(9) showed that SCH would produce phenocopies in *Drosophila* larvae.

Micromelia has been produced by sulfanil-

amide and eserine sulfate(10), insulin(11), thallium(12), boric acid(13), and pilocarpine (14). Lyons and Insko reported chondrodystrophy in the chick embryo produced by manganese deficiency in the diet of the hen (15). Nicotinamide has been used to overcome the teratogenic effects of insulin(16), sulfanilamide(17), eserine sulfate(18), and pilocarpine(14). In the present studies, administration of 5 mg nicotinamide concurrently with 1-3 mg of SCH in no way modified the characteristic syndrome in the chick embryos.

Antagonistic effects of vit. B_6 for hydrazides in bacterial and tumor growth have been discussed(19). Yet pyridoxal • HCl and pyridoxine • HCl in 1 mg quantities administered with 2 mg SCH did not alter the results in the present work. A condition similar to the SCH effects resulted in embryos from hens on a biotin-deficient diet(20). Nevertheless, when administered to the embryo treated with 1-2 mg of SCH, biotin up to 2 mg/egg did not alter the production of abnormalities described in this paper. Similarly, riboflavin in quantities up to 1 mg/egg did not affect the action of SCH.

Summary. Semicarbazide • HCl when injected into the embryonated White Leghorn egg at 4-6 days produced shortened and malformed lower beak and bent tarsometatarsal and tibiotarsal bones. The embryos were most sensitive to this injection at 6 days of incubation. Gross and histological effects were quite marked 48 hours after injection. Nicotinamide, pyridoxal, pyridoxine, biotin, and riboflavin administered simultaneously with SCH did not overcome the effects. Different effects on bone and soft tissues were produced with 1-phenylsemicarbazide. p-Hydrazinobenzoic acid, but not benzoic hydrazide, produced abnormalities very similar to those following SCH treatment. Only a small incidence of skeletal defect resulted from administration of thiosemicarbazide.

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Effect of Ingested Vanadium on Cholesterol and Phospholipid Metabolism in the Rabbit.* (22551)

JOHN T. MOUNTAIN, FULTON R. STOCKELL, JR. AND HERBERT E. STOKINGER.
Occupational Health Field Headquarters, Public Health Service, Cincinnati, O.

The metabolic effects of vanadium have been investigated as part of a study of the toxicology of vanadium relative to industrial exposures. Lipid metabolism was given particular scrutiny because the following isolated reports strongly indicated involvement of this metabolic component: (a) Congestion and fine droplets of fat in the liver have been produced by administration of vanadium compounds to rats(1). (b) Reduction in fat and lipid content of adrenal cortex occurred at the same time. (c) Addition of μg quantities of vanadium acetate or metavanadate to minced liver slices caused increased oxygen uptake: the fatty acid moiety was the reactive part of the substrate molecule(2). (d) In man, intramuscular injection of sodium tetravanadate resulted in increased catabolism as indicated by increased output of all nitrogen, sulfur, and phosphorous constituents determined in the urine(3). (e) Ingestion of vanadium pentoxide at dietary levels beginning at 100 ppm vanadium caused lessening of cystine content of rat hair(4). (f) While the present work was in progress, a report appeared stating that vanadium inhibited *in vitro* synthesis of cholesterol by rat liver cells(5).

Methods. 2.5 kg albino rabbits were used, 5 to a group, for each diet. The stock diet for control groups consisted of Purina Rabbit Chow Checkers. Test diets consisted of stock diet pellets coated with dextrin and the desired additives. Test diets were prepared by sprinkling 2.5 kg lots of stock diet with a blended corn dextrin suspension containing vanadium pentoxide and cholesterol additives. About 500 ml of distilled water containing 25-50 g of dextrin and the required amounts of additives were homogenized in a Waring Blendor. The creamy suspension was applied from a separatory funnel and stirred into the pellets. The coated pellets were air-dried 1 or 2 days. Vanadium was used as vanadium pentoxide which is of particular interest in industrial hygiene. Cholesterol was first converted to the amorphous form as recommended by Popjak(6). Coated pellets lost some vanadium, but not cholesterol, during handling, and rabbits would not eat the fine material. In practice 20-30% excess of vanadium over the calculated amount produced the desired vanadium content of large food pellets which the rabbits would eat. Spectroscopic analysis was used in checking diets for proper vanadium content(7). Food was given *ad libitum*: the amount consumed by each rabbit was recorded twice weekly.

Sampling and analysis. Blood was drawn from the marginal ear vein into tubes containing 1-2 mg Manuronate® anti-coagulant; specimens were taken at weekly or biweekly intervals. Food was withheld 16 hours prior to bleeding. Duplicate analyses were carried out: where only total cholesterol was determined, the method of Abell was used(8). Free and total cholesterol were determined by the digitonin-anthrone method (9, a, b); Zak's procedure(12) was used to obtain data in Tables II and III. Phospholipid analyses were carried out using a modified Youngburg method(10); the phosphomolybdenum blue was developed by Allen's method(11). The animals were killed by cardiac injection of air after final blood samples were drawn. At autopsy, tissue specimens were taken for analysis and pathologic examination. Liver samples were ground with sand and 12 parts anhydrous sodium sulfate and extracted with chloroform in a Soxhlet extractor. The chloroform extracts were evaporated at low heat on a steam bath, taken up in petroleum ether, filtered and the lipid content of the extracts determined by weighing after warm water bath evaporation. The lipid residue dissolved in 100 ml petrol-

* Presented before American Chemical Society, Minneapolis, Sept. 14, 1955.

TABLE I. Effects of 100 ppm Vanadium, as V_2O_5, with and without 1% Cholesterol on Livers and Liver Lipids of Rabbits in 4 Groups.

Diet	Lipid in liver, %	Total cholesterol, mg	Free cholesterol, mg	Phospholipid, mg	Vanadium in liver, μg
1: Control			*	†	
Mean	2.6	323	155	1840	2.0
Range	(2.0–3.1)	(168–826)	(128–176)	(1410–2152)	(.9–2.5)
2: 100 ppm V			*	†	
Mean	4.5	230	52	1100	54
Range	(2.4–8.9)	(175–286)	(20–89)	(495–1553)	(39–82)
3: 1% cholesterol					
Mean	9.3	3945	657	2170	2.3
Range	(8.1–10.9)	(2726–5425)	(397–1310)	(1540–3205)	(.9–3.3)
4: 1% chol + 100 ppm V					
Mean	8.9	2668	269	1634	66
Range	(3.3–14.1)	(651–3575)	(165–427)	(985–2579)	(13–112)

* Differing significantly, $P < .005$ by "F" test.
† *Idem* $P < .05$

eum ether was aliquoted for determinations of free and total cholesterol and phospholipid.

Diets. Composition of diets and duration of feeding are given in the Tables. Fig. 1 and Table I give results of the preliminary experiment to determine if vanadium affects plasma and liver cholesterol content. Fig. 2 shows data obtained in determining minimal levels of dietary cholesterol and vanadium necessary to produce notable differences among the groups compared. Tables II and III show effects of vanadium on plasma and liver cholesterol and phospholipid levels, both on continued cholesterol feeding with and without vanadium, and on these components when cholesterol was withdrawn from the diet, but vanadium intake continued after high plasma cholesterol levels had been attained. Some exceptions to the general procedure occurred: the rabbits used to obtain the data in Fig. 1 and Table I were not from the same source as

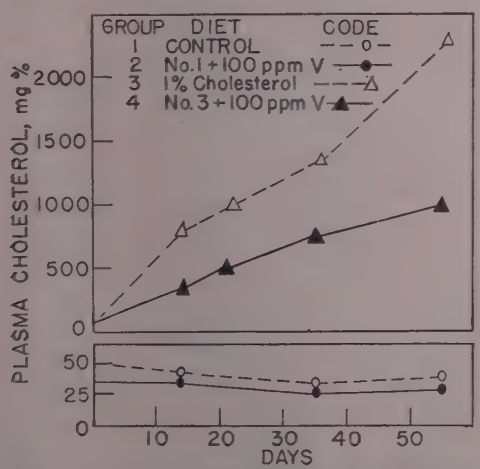

FIG. 1. Effects of feeding 100 ppm vanadium, as V_2O_5, with and without 1% cholesterol, on rabbit plasma cholesterol levels. Points represent averages of 5 animals.

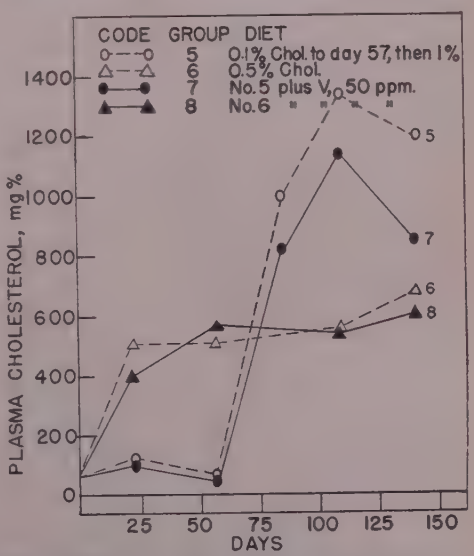

FIG. 2. Effects of feeding 50 ppm vanadium, as V_2O_5, with various amounts of cholesterol, on cholesterol content of rabbit plasma. Points represent averages of 5 animals.

TABLE II. Cholesterol and Phospholipid Content of Rabbit Plasma when 1% Cholesterol Was Fed with and without 100 ppm Vanadium as V_2O_5. Cholesterol omitted from diet of half the animals in each group after 50 days.

Diet group		Total cholesterol, mg %				Free cholesterol, mg %				Phospholipid, mg %				
↓ Days fed→	0	27	41	55	68	0	27	41	55	68	0	27	41	55	68
9: 1% chol															
Mean	51	*1100	*1688	§2198	*1634	13	*268	†412	511	*350	98	*432	*525	‡617	§545
Range	31–70	846–1387	1249–2147	1654–3168	1471–1843	9–21	173–377	323–521	387–662	277–400	88–110	342–545	435–672	445–807	390–685
10: No. 9 + 100 ppm V															
Mean	49	*818	*1204	§1443	*1123	13	*183	†325	442	*213	98	*315	*393	‡396	§375
Range	34–73	708–974	915–1690	1320–1584	936–1258	8–22	165–215	299–362	345–493	152–255	68–130	255–400	345–505	325–468	283–512
9-0: Chol omitted															
Mean				622	339				116	†106				258	§214
Range				528–781	183–464				92–136	77–128				218–288	158–255
10-0: Chol omitted															
Mean				636	286				110	†70				220	§167
Range				581–739	196–345				92–127	51–78				175–260	133–207

Corresponding pairs of means differing significantly by "F" test: * $P < .005$. † $P < .01$. ‡ $P < .025$. § $P < .05$.

Note: The groups compared are 9 *vs* 10, and 9-0 *vs* 10-0; although 9 and 10 are selectively reduced in number after day 50, they still represent equivalent samples with respect to each other, referred to the start of the experiment.

TABLE III. Effects on Growth, Liver and Liver Lipids of Rabbits when 1% Cholesterol Was Fed with and without 100 ppm Vanadium, as V_2O_5. Cholesterol omitted from diet of half the animals in each group after 50 days.

Diet group		Food consumed, g		Body wt change, g	Liver wt, g	$100 \times \frac{\text{Liver wt}}{\text{Body wt}}$	% lipid in liver	Total cholesterol, mg/100 g	Free cholesterol, mg/100 g	Phospholipid, mg/100 g	Vanadium in liver, μg/100 g
		All–68 days	Final 18 days								
9: 1% Chol	Mean	8004	1524	949	144	4.3	9.7	*4627	1066	3849	
	Range	7677–8578	1355–1728	764–1202	132–152	4.1–4.4	7.4–13.6	2982–6687	794–1312	2485–5962	
10: No. 9 + 100 ppm V	Mean	8216	1616	1014	156	4.5	9.8	*4217	846	2687	38
	Range	7383–8762	1033–1965	841–1163	132–176	3.8–5.4	8.1–10.7	3470–5643	712–1100	1330–4062	33–54
9-0: Chol omitted	Mean	8103	1619	1019	136	4.1	7.5	3046	759	1884	
	Range	7727–8578	1442–1895	802–1161	115–142	3.4–5.4	5.8–8.4	2410–3540	455–1008	1425–2697	
10-0: Chol omitted	Mean	8112	1617	1084	118	3.5	7.7	2635	653	1746	45
	Range	7732–8657	1475–1840	848–1257	104–156	3.2–4.3	5.4–9.0	1471–3072	378–914	1320–2212	22–84

* Corresponding pairs of means differing significantly by "t" test, $P < .05$.

Comparisons are made between corresponding groups, *i.e.* 9 *vs* 10, and 9-0 *vs* 10-0.

the others; Tables II and III include data from animals on diets partially restricted to maintain uniform food consumption.

Results. Fig. 1 shows total cholesterol levels in the plasma of rabbits receiving vanadium with the stock diet were slightly, but not significantly, lower than those of the control group. However, when 1% cholesterol was fed, the addition of vanadium produced highly significant reductions in the total cholesterol of the plasma. Thus on day 55, Group 3, fed 1% cholesterol, averaged 2267 mg% total cholesterol in the plasma, while Group 4, which received the same diet with vanadium added, had an average level of but 896 mg%. When cholesterol was fed, the free cholesterol of the plasma also showed differences attributable to vanadium: an average of 659 mg% free cholesterol in the plasma of Group 3 rabbits (no vanadium) compared to 320 mg% for Group 4 (vanadium-fed). Table I shows that the addition of vanadium to the stock diet resulted in reduction of the average free and total cholesterol and phospholipid content of the liver of this group (Group 2) compared to the stock diet controls (Group 1). Similarly, the average quantities of these 3 substances in the livers of the animals fed 1% cholesterol along with vanadium (Group 4) are lower than the amounts found in the comparable group (Group 3) which received no vanadium. Food consumption, weight gains, liver weights and liver weight/body weight ratios were not significantly different for comparable groups.

In Fig. 2, it should be noted that Groups 5 and 7 are compared, so far as the plasma is concerned, at 2 levels of dietary cholesterol. It is evident that 50 ppm V had but little, if any, effect for the first 57 days when these groups were receiving 0.1% cholesterol. Subsequently, raising the cholesterol to 1% of the diet, definitely brings out differences in the plasma levels in these groups. On day 110 (53 days on 1% cholesterol), total cholesterol averaged 1333 mg% for Group 5, 848 mg% for Group 7, ($P<.05$). Correspondingly, free cholesterol was 507 mg% against 354 mg%: phospholipid levels followed this pattern to a lesser degree (390 *vs* 353 mg%). Groups 6 and 8 appear to show little, if any, real differences associated with the presence or absence of 50 ppm vanadium when cholesterol is fed as 0.5% of the diet. The livers of comparable groups showed slight differences associated with feeding vanadium under the conditions of this experiment.

Table II shows that by the 27th day 100 ppm dietary vanadium produces significant effects on all the plasma lipids in the table. These effects persisted for the duration of the experiment where the regimen was constant throughout. The peak followed by a drop (eventually leveling off) of plasma levels in groups 9 and 10 at about 55-68 days follows the pattern noted by Popjak(6). In the case of the animals from whose diet cholesterol was omitted on day 50, *i.e.* groups 9-0 and 10-0, the table shows the plasma lipids returned to near normal levels when vanadium was in the diet, whereas the rate of decline of the plasma lipids following withdrawal of cholesterol proceeds at a definitely slower pace when vanadium is not fed. The more rapid disappearance of pre-formed cholesterol in the presence of vanadium supports the oxidation-catalyst mechanism. Table III shows that food consumption of comparable groups was virtually identical: the vanadium groups consumed slightly more food, consequently more cholesterol. This tends to rule out lower food consumption as a reason for lower cholesterol levels in the vanadium-fed animals. Average concentrations of liver lipids are lower for vanadium animals: in most cases they are nearly great enough to be significant at the 5% level.

Pathologic examination of the tissues of the rabbits revealed that all animals which ingested cholesterol had atheromas of the coronary arteries and aortas, as well as lipid deposition in the heart. The liver sections exhibited foamy infiltration. Adrenals contained abundant lipid material and some animals had considerable lipid in the intestinal mucosa. Of possible significance was the absence of intracellular lipid deposition in the kidney tubules of vanadium treated rabbits, while 50% of the animals not given vanadium showed lipid deposition in the tubular epithelium. Marked

differences in appearance of vanadium and nonvanadium livers were evident. The color of the vanadium livers was similar to normal livers, while livers of rabbits fed cholesterol without vanadium were much lighter. The aortas of groups in Tables II and III were photographed in color and the color slides were examined and graded as to extent of plaque formation. Without knowledge of the diet the particular animal received, grouping the aortas according to severity of plaque formation served to separate them in relation to diets: the vanadium-fed animals invariably had less extensive areas of aortal plaques than did those receiving cholesterol alone. It may be noted here, that Curran has recently reported a reduction in aortal cholesterol following administration of vanadium to rabbits (15).

Discussion. The lower plasma cholesterol and phospholipid accompanied by lower free cholesterol and phospholipid in the liver of vanadium animals may result from vanadium either accelerating metabolism by the liver or by inhibiting cholesterol synthesis. Inhibition of synthesis has already been demonstrated *in vitro*(5): accelerated metabolism is suggested by the fact that with low vanadium (50 ppm, Fig. 2), it was found that no appreciable effects on plasma lipids were observed when cholesterol levels were low (0.1, 0.5%). This implies interaction and could be explained on the basis that vanadium is reacting chemically with cholesterol, perhaps as an oxidative catalyst. The minute amounts of vanadium (16-68 μg) in the liver support this concept. Vanadium is rapidly excreted after absorption(13): it is probable that less than 50 ppm of vanadium in the diet does not maintain enough vanadium in the liver to have measurable effects on the cholesterol passing through. Some evidence as to the form of cholesterol acted upon by vanadium appears in Table I: when no extra cholesterol was fed, the free cholesterol of the liver averaged only one-third that of nonvanadium controls. However, this could be explained on the basis of inhibition of cholesterol synthesis. The significant reduction in phospholipid paralleling the low free cholesterol in this case is consistent with the observations of Popjak(6) that free cholesterol controls phospholipid mobilization. Inhibition of protein synthesis resulting in lowered lipoprotein formation(14) does not appear to be a factor in the lowering of the plasma cholesterol levels; had this occurred, cholesterol would have accumulated in the liver: on the contrary, both plasma and liver cholesterol levels were reduced by vanadium. Finally, as judged by weight gains, liver weight-body weight ratios and examination of tissues and organs, vanadium, at the levels fed in this experiment, had no adverse effects on the animals.

Summary. 1. Vanadium, in the form of vanadium pentoxide, added to a standard diet, at a level of 100 ppm of the element lowered free cholesterol and phospholipid content of the liver of rabbits, while plasma cholesterol showed no significant changes. 2. Elevation of free and total cholesterol levels of plasma was restricted significantly by the addition of 50 ppm vanadium to a 1% cholesterol diet. Phospholipid levels also were maintained at lower levels, on the average, although not uniformly. 3. When rabbits were fed cholesterol to raise the plasma levels of cholesterol and phospholipid, and then the cholesterol was omitted from the diet, it was found that the presence of vanadium in the diet produced a faster rate of return to normal than occurred when no vanadium was furnished. 4. The mechanism by which vanadium restricts elevation of plasma cholesterol appears to involve both inhibition of cholesterol synthesis and accelerated catabolism of cholesterol. The latter may occur through a catalytic process.

The substantial contributions of Dr. Herbert Lansky, pathologist, Robert G. Keenan, spectrochemist, Wm. D. Wagner, toxicologist, Tetsuo Shimamoto, statistician and Laryl Lee Delker, physical science aide, to this work are gratefully acknowledged. We wish to express our appreciation to Drs. Joseph H. Bragdon of the National Institutes of Health, and George L. Curran of the University of Kansas Medical Center for their comments and helpful suggestions.

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Quantitative Lipoprotein Studies in Normal and Abnormal Subjects Using Combined Electrophoretic and Chemical Technics.* (22552)

FREDERICK R. BROWN, JR.,† GEORGE D. MICHAELS, AND LAURANCE W. KINSELL.
(With the technical assistance of Sadie Smyrl and June Bilisoly.)
The Institute for Metabolic Research of Highland Alameda County Hospital, Oakland, Calif.

The general problem of atherosclerosis may be considered to have 3 major facets: 1) Elucidation of pathogenesis of atherosclerosis; 2) establishment of diagnostic laboratory procedures; 3) evaluation of methods to prevent or treat the disease. The relationship of disturbances in serum lipids to pathogenesis of atherosclerosis has been studied extensively. Interest has centered on distribution of cholesterol, phospholipids, and neutral fat between the two major lipoprotein complexes, the so-called alpha and beta lipoproteins. Barr *et al.*(1) demonstrated a definite correlation between percent distribution of cholesterol in alpha and beta lipoprotein complexes and the presence of clinically evident atherosclerosis and diseases predisposing to atherosclerosis. Cohn fractionation, the method used by these investigators for separation of alpha and beta lipoprotein complexes, is technically difficult, expensive and time consuming.

Several reports have dealt with separation of alpha and beta lipoproteins by paper strip electrophoresis and subsequent semiquantitation of total lipid staining material in the alpha and beta fractions, by means of scanning devices(2). Determination of total lipids in lipoprotein complexes by staining, elution, and colorimetric determination has been reported(3). Kunkel and Slater(4a) and Nikkila(4b) have attempted chemical quantitation of serum cholesterol and phospholipid after paper electrophoretic separation, and subsequent elution of alpha and beta lipoprotein entities.

The present report deals with a method for the separation of alpha and beta lipoprotein complexes by paper strip electrophoresis, and subsequent quantitation of total lipid, total cholesterol, and phospholipid in each of these complexes.

Method. Two 6.5 cm wide Whatman No. 3MM filter paper strips are moistened in a barbital buffer solution (pH 8.6, ionic strength 0.05%). They are then placed in each of 3 plastic boxes having the dimensions of 24 x 18 x 6.5 cm. Each end of the paper strips is immersed in a 1.5 liter buffer reservoir placed at the end of the boxes. 0.20 ml

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† Alameda County Heart Assn., Fellow, 1955-56.

of serum is applied by pipette evenly across the width of the paper strip, except for a margin of 1.0 cm at each side. Electrophoresis is carried out for approximately 18 hours, maintaining a constant potential of 80 volts. After this process is complete, the strips are removed from the boxes and dried, then fixed and stained in a solution of bromphenol blue, prepared as follows: to 400 ml of 2% acetic acid add 0.2 g bromphenol blue; mix; add 4.0 g of bichloride of mercury and shake; add slowly dropwise strong hydrochloric acid until the solution changes from red to orange. To this stain, add 45 ml of formaldehyde solution (approximately 37%) and mix. This stain is good for several weeks. After staining and washing in 2% acetic acid, the strips are cut into 2 portions between the alpha and beta components. This effectively separates the albumin and alpha globulins from the gamma and beta globulins, respectively, and includes all stainable material. The 2 portions of each strip are dried in room air, and subsequently treated identically. Each is cut into small pieces and placed in 10 cc of boiling water. This is evaporated nearly to dryness, and then eluted with a hot solution containing equal parts of acetone and ethyl alcohol. The eluates from the 3 corresponding paper portions are combined. The solutions derived from the "alpha and beta lipoprotein complexes," respectively, are divided into 3 aliquots for determination of total cholesterol, lipid phosphorus and total lipid (in duplicate). *Cholesterol determination*: The first aliquot is evaporated to dryness, then taken up in a total volume of 5 ml of acetone alcohol. After hydrolysis of the esterified cholesterol with 5 normal potassium hydroxide, total cholesterol is determined. In this laboratory a modification of the Schoenheimer-Sperry technic is used(5). *Lipid phosphorus determination*: The second aliquot is evaporated to dryness and then dissolved in approximately 10 ml of acetone alcohol, and transferred to a pyrex tube. It is again evaporated to dryness and digested with concentrated sulfuric acid. Digestion is completed with 28% hydrogen peroxide (Superoxal). Determination of phosphorus is then carried out by the method of Fiske and Subbarow(6), and reported as lecithin (x 25). *Total lipid determination*: The third aliquot is evaporated to dryness and extracted with petroleum ether. Total lipid is determined, using the method of Bragdon(7). Arbitrarily, we have reported the difference between the sum of phospholipid plus cholesterol, and total lipid, as neutral fat. Total cholesterol, phospholipid, and total lipid are determined macrochemically on whole serum and the recovery of lipid from the paper strips compared with these totals.

TABLE I. Recovery of $\alpha + \beta$ Lipid Entities (Expressed as Percent of the Same Lipids in Whole Serum).

	Mean % recovery	Stand. dev.
Cholesterol	98.9	4.4
Phospholipid	101.5	6.6
Total lipid	98.2	5.2

Results. Recovery. Data obtained from twenty sera from 14 individuals are included in this report. (Table I).

Normal and abnormal sera. Fig. 1 shows the percent concentration of total lipid, neutral fat, total cholesterol and phospholipid present in the "alpha lipoprotein" (in relation to serum levels of the same lipids) from 6 normal young adult females, 3 normal young adult males, and 5 patients with atherosclerosis or diseases predisposing to atherosclerosis. These patients include a male with diabetes of 18 years' duration associated with hypercholesterolemia; a young female with diabetes of 4 years' duration, with no clinical evidence of vascular disease; a 54-year-old male with angina pectoris and gout; a middle aged male with familial hypercholesterolemia with no clinical evidence of vascular disease; and lastly, a young female with familial hypercholesterolemia with no clinical evidence of vascular disease.

Findings to date show: 1) No obvious separation of normals from abnormals in terms of total lipid or neutral fat distribution; 2) overlapping of normals and abnormals in terms of total cholesterol distribution; 3) sharp separation of normals from abnormals, in regard to phospholipid distribution.

Under way at the present time are com-

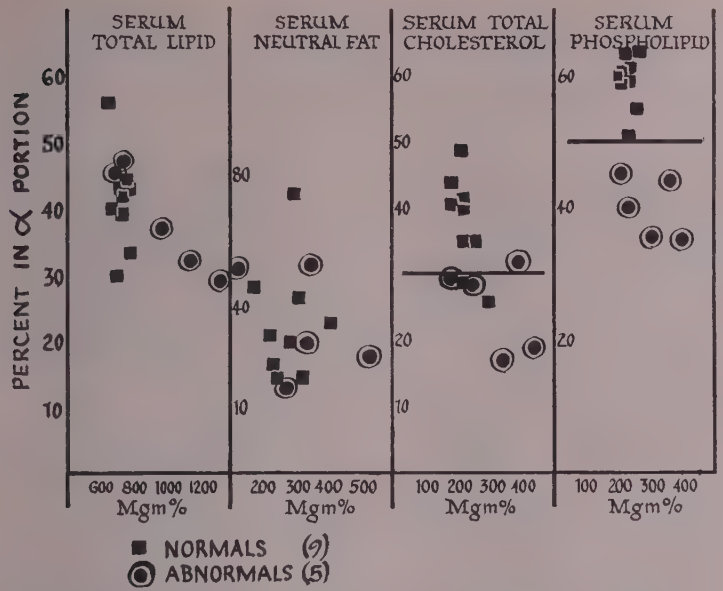

FIG. 1. Serum "α lipid" constituents in normal and abnormal subjects.

parative studies using paper electrophoretic separation and Cohn fractionation in identical sera.

Summary. 1) A method for paper electrophoretic separation and quantitative determination of serum alpha and beta lipoprotein neutral fat, cholesterol and phospholipid, respectively, is described. Studies in 9 normal and 5 abnormal subjects are included. 2) The sums of total lipid, cholesterol and phospholipid recovered after electrophoretic separation of alpha and beta lipoprotein are compared with those values determined macrochemically in untreated sera. Separation of abnormal from normal individuals appears to have been achieved in this small series in terms of distribution of phospholipid, and possibly of cholesterol, in the alpha and beta lipoprotein complexes.

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Effects of Hypophysectomy and Growth Hormone on Ploidy Distribution and Mitotic Activity of Rat Liver.* (22553)

H. S. Di Stefano and H. F. Diermeier. (Introduced by J. Tepperman.)
Departments of Anatomy and Pharmacology, State University of New York, Syracuse, N. Y.

In a recent cytophotometric study of the effects of growth hormone on rat liver protein and nucleic acids, Di Stefano, *et al.*(1) reported that, within a given class of ploidy, the deoxyribose nucleic acid (DNA) content of liver nuclei was not affected either by hypophysectomy or by the administration of a purified growth hormone preparation. However, differences were noted in ratios of diploid and tetraploid nuclei in livers of hypophysectomized rats as compared with hypophysectomized rats treated with growth hormone and intact control animals. The diploid/tetraploid ratios observed in the hypophysectomized animals were found to be consistent with the pattern seen in younger animals, while the pattern in hypophysectomized animals treated with growth hormone was characteristic of that found in older intact animals.

In an attempt to arrive at a better understanding of the mechanisms responsible for these differences in ploidy patterns, it seemed pertinent to investigate the ploidy ratios in livers of hypophysectomized rats during the first 8 days postoperatively, and to correlate these with some index of mitotic activity in the same livers.

Methods. Hypophysectomized and intact male Sprague-Dawley rats were purchased from the Hormone Assay Laboratory, Chicago, Ill., separated into groups containing 10 animals per group and maintained on a diet of canned dog food which was fed *ad libitum*. These groups were treated as follows: 1. One group of intact control animals (average body weight on arrival, approximately 75 g) was sacrificed when their average body weight reached that of the hypophysectomized animals. This group of animals (average body weight, 93.7 $\pm$ 1.2 g at the time of sacrifice) was comparable to the hypophysectomized groups on the day of the operation and therefore constitutes what might be called the initial intact control group. 2. Five groups of hypophysectomized animals were sacrificed 1, 2, 4, 6 and 8 days postoperatively. The average weights of animals in these groups were 93.2 $\pm$ 2.2, 93.0 $\pm$ 1.6, 91.1 $\pm$ 1.6, 91.0 $\pm$ 2.4, and 95.9 $\pm$ 2.5 g respectively. 3. One group of hypophysectomized rats (8 days postoperatively) weighing approximately 92.7 $\pm$ 1.6 g, were injected with 0.25 mg of a purified growth hormone preparation (Armour & Co., Lot. No. R491017)† twice daily by intramuscular injection for 7 days and sacrificed on the following day. At the time of sacrifice the average body weight of animals in this group was 137.0 $\pm$ 2.3 g. 4. One group of intact control animals weighing approximately 91.6 $\pm$ 0.6 g, were given 0.25 ml of 0.85% saline as before twice daily for 7 days and sacrificed on the following day. The average body weight of the animals in this group at the time of sacrifice was 142 $\pm$ 1.6 g. All animals were sacrificed by decapitation, and the livers removed rapidly and weighed. Small pieces were fixed immediately in 25% neutral formalin, washed in running tapwater for 48 hours, and embedded in paraffin. Measurements of relative amounts of DNA were made using the same procedures and apparatus described in a previous publication(1). Absorption measurements of Feulgen stained DNA were made at 560 millimicra, isolated from a tungsten source by means of a 250 mm Bausch and Lomb grating monochromator. Counts of mitotic figures per field of liver cells were also made and expressed as number of mitotic figures per 100 cells counted. The values are expressed as averages for 100 fields of liver per group of animals (10 fields of

* Supported by a grant-in-aid from Amer. Cancer Soc. upon recommendation of Committee on Growth of National Research Council.

† The purified growth hormone was generously supplied by Mr. Irby Bunding of Endocrine Research Division of Armour & Co.

TABLE I. Effects of Hypophysectomy and Treatment with Growth Hormone on the Mitotic Activity of Rat Liver.

Animal group (10 rats/group)	Mitotic activity (mean/100 cells counted ± S.E.)
Initial intact controls	1.94 ± .12
Hypophysectomized, 1 day	.18 ± .02
" , 2 days	.13 ± .02
" , 4 "	.14 ± .02
" , 6 "	.09 ± .02
" , 8 "	.08 ± .02
Final (8 day) intact controls	.48 ± .06
Hypophysectomized, 8 days; growth hormone, 7 days	.43 ± .04

liver per animal). Histograms showing the frequency distribution of relative DNA content of rat liver nuclei were constructed. Arbitrary units of DNA were calculated according to the method of Swift(2), for 25 liver nuclei in each of 10 rats per group. Since no DNA values below 5 were seen, an initial class interval of 5 to 5.5 was chosen. Each successive class interval was then increased 10% over the preceding interval. This method of presentation was selected because it was found that, if the class interval is kept constant, the representation of distribution in the 2 classes of nuclei (diploid and tetraploid) is not comparable. The method suggested‡ keeps the number of classes in the two ploidy ranges essentially the same.

Results. Fig. 1 shows a comparison of the ploidy distributions in livers of hypophysectomized rats compared with the patterns seen in intact control animals of the same weight. In the latter group of animals a predominance of diploid over tetraploid nuclei is seen. This characteristic ratio persists in livers of hypophysectomized rats as late as 8 days postoperatively.

A comparison of liver ploidy patterns of hypophysectomized, growth hormone treated hypophysectomized, and intact control rats may be seen in Fig. 2. Here one can observe: first, the ploidy distribution profile in livers of intact control rats shifts from one favoring a predominance of the diploid class to one in which the tetraploid class becomes the predominant one; second, the ploidy profile of the younger, lighter, rats persists as such after the removal of the hypophysis; and third, that treatment of hypophysectomized rats with a purified growth hormone preparation results in a liver ploidy distribution pattern essentially identical with that of the older, heavier intact control animals.

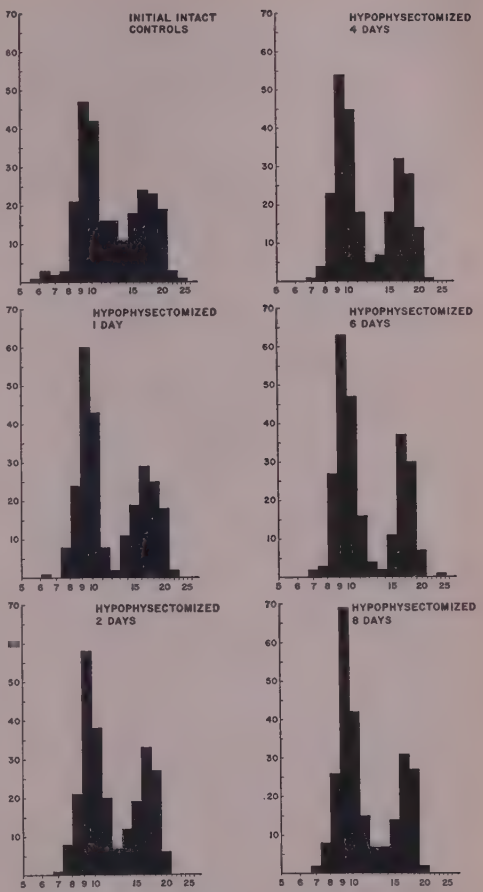

FIG. 1. Effect of hypophysectomy on ploidy pattern of rat liver. Ordinates represent frequency and abscissas arbitrary units of DNA.

The results of mitotic counts are summarized in Table I. Hypophysectomy results in a striking reduction of mitotic activity. As early as the first day after removal of the hypophysis the mitotic activity appears to be reduced by a factor of about 10. It may also be noted that the mitotic activity in livers of

‡ This method was suggested to us by Prof. C. L. Yntema.

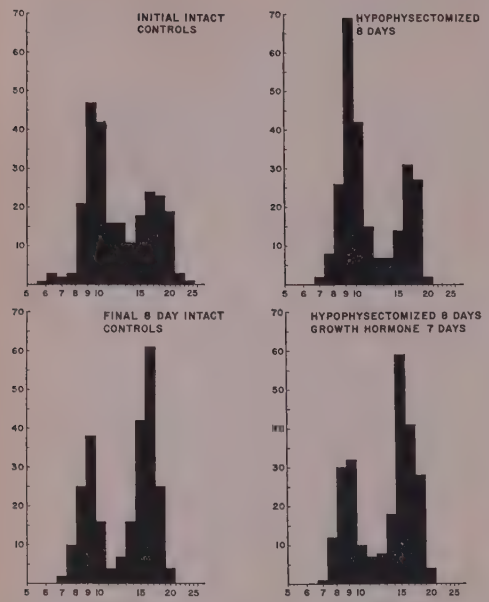

FIG. 2. Comparison of liver ploidy patterns of hypophysectomized, growth hormone treated hypophysectomized, and control rats. Ordinates represent frequency and abscissas arbitrary units of DNA.

heavier and older intact animals is normally lower than that of the initial intact controls and that growth hormone replacement in the hypophysectomized rat results in a mitotic activity consistent with the rate observed in intact control rats of comparable weight.

Table II contains a summary of the percentage distribution of diploid and tetraploid nuclei in all groups of rats (10 rats per group) represented in Fig. 1 and 2. It is clearly apparent that, within each class of ploidy, the distribution pattern of final intact controls and growth hormone-treated hypophysectomized rats are significantly different from all other groups at a confidence level of less than 1%. It is also evident that the distributions in hypophysectomized groups are not different from that of initial intact controls and that in hypophysectomized, growth-hormone-treated animals, the distribution does not differ from that of final intact controls.

TABLE II. Percentage Distribution of Diploid and Tetraploid Liver Nuclei of Control, Hypophysectomized, and Growth Hormone Treated Hypophysectomized Rats.

Animal group (10 rats/group)	Distribution of nuclei 2 N	4 N
Intact controls	58.7±1.3*	41.3±1.3*
Hypophysectomized, 1 day	60.0±1.1	40.0±1.1
" , 2 days	59.6± .9	40.4± .9
" , 4 "	59.2±1.0	40.8±1.0
" , 6 "	64.0±1.2	36.0±1.2
" , 8 "	63.6±1.3	36.4±1.3
Final intact controls	37.2±1.2†	62.8±1.2†
Hypophysectomized, growth hormone treated	40.0±1.1†	60.0±1.1†

* Mean ± S.E.

† Significantly different from all other values ($P<0.01$, Student's t test) but not significantly different from each other.

Discussion. In young rats the ploidy distribution profile of the liver shows a predominance of diploid over tetraploid nuclei. As the animal grows there is a reversal of this ratio brought about by a shift towards tetraploidy. The significance of the tetraploid shift that accompanies growth of the rat liver is not known. In any case, the present study reveals an easily detectable shift within the 8-day period of this experiment (Fig. 2).

The fact that the ploidy distribution pattern in the hypophysectomized rats resembles that seen in the initial intact controls strongly suggests that hypophysectomy results in an arrest of the tetraploid shift that occurs in the normal intact controls over a comparable period of time. In fact, observations made 21 days after hypophysectomy show a persistence of the more juvenile state of ploidy seen in the initial intact controls and in all untreated hypophysectomized groups. In light of these observations it is unnecessary to postulate (as was done in a previous publication) (1) a reversion to a lower state of ploidy following hypophysectomy. These data confirm and extend the results of previous studies (1) in which differences in ploidy profiles were found between hypophysectomized rats and larger intact controls of the same *age*. They are also consistent with observations on congenitally dwarfed mice made by Leuchtenberger, *et al.*(3).

The suggestion made by Bass, *et al.*(4) that there is an increase in diploid and octaploid nuclei must be considered in relation to the observations reported here. Their "increase" in the diploid population may be only

an apparent one, since they based their conclusions on a comparison of 141 g hypophysectomized rats with 226 g controls. The implication of a true increase cannot be accepted without question unless data on 141 g intact control animals are included for comparison. No "octaploid" shift of the sort described by Bass, *et al.*(4) was seen in this study, but this, too, may be attributed to the fact that the tissues of younger rats were examined. Most of the nuclei that contribute to the "octaploid" shift of Bass, *et al.*(4) are intermediate values between the tetraploid of about 17 and the octaploid of 34. In order to refer to this distribution as an octaploid shift it would be necessary to show that this pattern does not exist in the normal 141 g rat. In hypophysectomized animals, a similar appearance of large numbers of "intermediate" nuclei between the diploid and tetraploid groups was not seen in the present study. In fact, the intermediate values tended to diminish immediately after hypophysectomy; this finding is in agreement with the marked decrease in mitotic activity found at this time. Taken together, these data are not consistent with the construct that increased DNA synthesis (which is implicit in a shift towards octaploidy) is likely to occur after hypophysectomy.

The striking decrease in mitotic activity of the liver seen following hypophysectomy is consonant with the findings of Leblond and Carriere(5), who found a similar decrease in the mitotic activity of epithelium of crypts of Lieberkühn in the intestine of the rat after hypophysectomy. It should be noted that there was some mitotic activity after removal of the hypophysis in both liver and intestinal epithelium, suggesting that these tissues have an inherent mitotic activity which is independent of hypophyseal influences.

Administration of a purified pituitary growth hormone to a hypophysectomized rat can restore normal ploidy patterns, normal mitotic activity and normal concentrations of nuclear and cytoplasmic ribose nucleic acid and protein in liver cells(1,6,7). In addition the present observations on mitotic activity suggest that our previous finding of a significant increase in DNA per diploid nucleus after 2 days of growth hormone treatment (of 4 day hypophysectomized rats)(1), suggests that DNA synthesis may be among the consequences of growth hormone administration under these circumstances. Thus, growth hormone exerts an influence on the basic cellular biochemical machinery which is concerned with cell division and protein synthesis. The mechanisms by which this effect is exerted are quite unknown. In terms of reaction sequence, the biochemical locus of action of the hormone might be very remote from the phenomena described above. This is simply a reiteration of the view that the description of intracellular changes following the administration of a hormone does not necessarily signify that the hormone participated directly in effecting these changes.

Summary. 1) Ploidy distribution patterns and mitotic activity in livers of young rats have been studied at various times following hypophysectomy, and compared with distributions in livers of rats of comparable weight. 2) It was found, within the time limits of this experiment, that there is a shift of ploidy distribution from one in which the diploid class of nuclei predominates to one in which the tetraploid class predominates. During this period of time the mitotic activity in these same livers decreases. 3) Hypophysectomy results in an arrest of the ploidy distribution consistent with the ratios present at the time of hypophysectomy. This is accompanied by a marked reduction in mitotic activity. 4) Growth hormone replacement in the operated animals restores both ploidy distribution pattern and mitotic activity to that seen in intact controls of comparable weight.

The authors wish to acknowledge the technical assistance of Marilyn White and Marjorie Stoffel.

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A Component of Substance P Active in Releasing ACTH.* (22554)

W. W. SWINGLE, A. F. PARLOW, L. J. BRANNICK, AND WALTER BARRETT.
Biological Laboratory, Princeton University, Princeton, N. J.

Substance P is a potent smooth muscle stimulating factor first described by von Euler and Gaddum(1), and since studied by other investigators(2-7). The precise function of P is not clear, nor has the substance been well characterized chemically, although it is regarded by some as a complex polypeptide(1,5,8). The typical action of P upon smooth muscle, *e.g.*, the guinea pig ileum, is induction of a slow contraction starting after a short latent period. This effect is not abolished by atropine, antihistaminics or ganglionic blocking agents(4). Similarities in activity between the acid hydrolysate of P and that of vasopressin (Pitressin, Parke, Davis and Co.), now definitely known to contain a component which releases adrenocorticotropin, induced the writers to test P for comparable releasing activity.

Methods. Substance P,† prepared from the intestine of the horse, was tested for ACTH-releasing activity using the *in vitro* technic of Saffran *et al.*(9,10) and the Sayers adrenal ascorbic acid depletion test on 120-140 g male rats hypophysectomized by the parapharyngeal approach for 18-24 hours. Two minor modifications of the Saffran method were introduced in these experiments: (1) the rats from which the pituitaries were removed for incubation were gentled for several days before use and then quickly guillotined without anesthesia; (2) arterenol was not added to the incubation fluid since it served no useful purpose.

Results. Substance P was hydrolyzed by boiling 1.5 hours in 2.2 N HCl, using the method previously described for hydrolysis of vasopressin(11,12). The results of the tests, shown in Table IA, demonstrate that both hydrolyzed and nonhydrolyzed P exhibit marked ACTH-releasing activity amounting to -106 ± 11.5 and -110 ± 12.3 mg % adrenal ascorbic acid depletion. Thus acid hydrolysis does not seem to destroy the activity of P. Equivalent amounts of incubation fluid from the Warburg flasks containing control pituitary glands and given I.V. to 18 hypophysectomized rats induced negligible ascorbic acid decline. Nonhydrolyzed P was also tested for its *in vivo* ACTH-releasing action on intact rats whose ACTH output had been inhibited by subcutaneous injections of 10 mg DCA plus 10 mg of the free alcohol of hydrocortisone 18 hours previously. P was administered I.V., to 6 rats in a dose of 0.25 mg one hour before removal of the second adrenal for the ascorbic acid test. The results were essentially negative, but should be repeated when purer preparations are available. Since P is destroyed by strong acid at boiling temperature, whereas the hydrolysate retains its ACTH-releasing activity, it seems evident that the releasing agent is not the same substance designated as P by von Euler, Gaddum and others, but is quite a different entity.§ To avoid confusion in terminology the ACTH-releasing component of the P complex will be referred to hereafter simply as P_1 until it is more precisely characterized chemically.

* Part of the expenses of this investigation was defrayed by Sharp and Dohme, Division of Merck and Co., West Point, Pa., and Ciba Pharmaceutical Products Co., Summit, N. J.

† We are indebted to Drs. D. A. McGinty and J. J. Pfiffner of Parke, Davis and Co. for generous supplies of Pitressin and Substance P.

§ Chromatographic fractions (obtained from Merck & Co.) also show separation of releasing agent and P.

TABLE I. Adrenocorticotropin Releasing Substances.

	Amt material used in Sayers assay	No. of rats	Ascorbic acid depletion, mg %*
A. Release of ACTH from anterior pituitaries *in vitro* by Substance P, 0.67 mg P in 0.5 cc incubation fluid/Warburg flask			
		Hypophysectomized	
P—Nonhydrolyzed	.20 ml Warburg fluid	17	−110 ± 12.3†
Control	*Idem*	11	− 17 ± 7.3
P—Hydrolyzed	"	7	−106 ± 11.5†
Control	"	7	− 19 ± 13.6
Pitressin—Hydrolyzed	"	37	−111 ± 18.6†
Control	"	43	− 23 ± 6.6
B. Ineffectiveness of Substance P when infused I.V. in hypophysectomized rats			
P—Nonhydrolyzed	5.35 mg/rat	5	+ 22 ± 19.2

* Mean ± stand. error. † Differs significantly from controls ($P < 0.01$).

Substance P (containing P_1) is without effect on the adrenals of animals lacking pituitary glands when infused I.V., and differs in this respect from pitressin, since the latter when administered I.V., in very large doses (300 units) to hypophysectomized rats is toxic and apparently is contaminated with sufficient ACTH to markedly deplete adrenal ascorbic acid. In our experience such massive dosage induced a sharp decline in ascorbic acid amounting to —128 ± 3.9 mg %. Nonhydrolyzed P, rich in P_1 factor, does not exhibit strong pressor activity, hence is devoid of toxicity and can be infused I.V. into hypophysectomized rats in large doses. Five animals whose pituitary glands had been extirpated 18-24 hours earlier, were given 5.35 mg of P by vein. No signs of toxicity appeared nor was the adrenal ascorbic acid depleted (Table IB). Incubation of 0.67 mg of this same material in a Warburg flask containing pituitary glands released ACTH which greatly decreased the adrenal ascorbic acid of hypophysectomized rats when the incubation fluid was infused.

Experiments on the Guinea Pig Ileum. The suggestion has been made(5) that Substance P may consist of more than one polypeptide. The following experiment indicates that there are probably 3 factors in P, each possessing different pharmacological properties. Hydrolysis with 2.2 N HCl destroys the agent which stimulates the slow increase in tone of the isolated guinea pig ileum but does not inactivate the ACTH-releasing factor in P. Hydrolysis also frees a material which exhibits physiological properties identical with those of the histamine-like substance obtained by similar hydrolysis of pitressin(11,12). Thus nonhydrolyzed P when tested on the ileum, induces a slow increase in tone (Fig. 1), which is not antagonized by pyribenzamine or atropine. The hydrolysate given in the same dosage evokes an altogether different response, indistinguishable from that elicited by histamine and which is abolished by pyribenzamine (Fig. 1). Since both hydrolyzed and nonhydrolyzed P are ACTH releasers, the striking difference in the gut response to acid and non-acid treated material affords additional evidence that P itself is not the re-

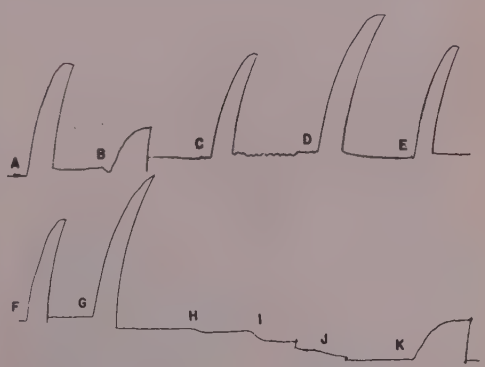

FIG. 1. Isolated ileum of guinea pig. Doses given per cc of bath fluid. A, C, E, F: Histamine base 0.015 γ/cc. B and K: Substance P, 67 γ/cc. D and G: Hydrolyzed Substance P, 67 γ/cc. H: Pyribenzamine 0.5 γ/cc. I: Hydrolyzed Substance P, 67 γ/cc. J: Histamine base 0.015 γ/cc. K: Nonhydrolyzed Substance P.

leasing agent. This is an uncharacterized substance tentatively designated here as P_1. To date the ACTH-releasing factor in the hydrolysate of P has not been separated from the histamine-like component. However, it is probable that this latter substance is not concerned with ACTH release because a similar, if not identical, pharmacologically active agent occurring in pitressin hydrolysates can be eliminated (unpublished data) from ACTH-releasing fractions without loss of potency by the latter. This separation is readily accomplished by distribution in the Craig Counter Current apparatus using a suitable solvent system.

The hydrolysate of P was tested on the blood pressure of the etherized-atropinized cat and the results compared with those elicited by histamine and 5-hydroxytryptamine. The vasodepressor action of P-hydrolysate was less evident than that shown by equal amounts of hydrolyzed pitressin(11). The fall in blood pressure was comparable to that evoked by histamine but quite unlike that induced by 5-hydroxytryptamine (Fig. 2, A-D).

Discussion. The data presented by Guillemin‡ and the present writers‡ render it probable that the ACTH-releasing agent now definitely known to be associated with both Substance P and commercial vasopressin, either as a contaminant or possibly a fragment of an originally larger protein molecule, are one and the same entity. This offers interesting possibilities in view of the widespread distribution of P (including P_1), since it is especially prevalent in the nervous system, both central and peripheral. According to Pernow(4), Amin *et al.*(7) and others, P occurs in the hypothalamus in greater concentration than in most other tissues. The fact that P (P_1) releases ACTH is significant, since it has been suggested(4,6,7) that this physiologically potent substance may be the chemical transmitter liberated by the first sensory neurones. Such an hypothesis if definitely established would perhaps afford a rational explanation for the extremely rapid release of ACTH under conditions of stress.

‡ Reported at Annual Meeting of Fed. Soc. for Exp. Biol. and Med., April 1956, Atlantic City, N. J.

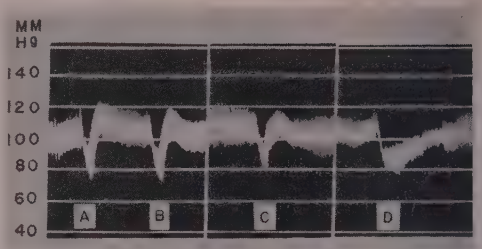

FIG. 2. Cat, 2.4 kg, atropinized, etherized, carotid blood pressure. A: Histamine base 0.6 γ/kg I.V. B: Histamine base 1.8 γ/kg I.V. C: Hydrolyzed Substance P, 10.4 mg/kg I.V. D: 5-hydroxytryptamine 20 γ/kg I.V.

Although the exact chemical nature of the active ACTH-releasing component of P and pitressin is unknown, evidence exists that they are probably identical and can be separated as distinct entities from (1) pitressin; (2) Substance P, and (3) the histamine-like material which makes its appearance in acid hydrolysates of both P and pitressin.

Summary. Substance P, prepared from horse's intestine, is rich in a component which is highly active in releasing ACTH from pituitary glands *in vitro*. This factor is not P itself but is an accompanying material of unknown nature which can be separated from P by strong acid hydrolysis and shown to contain all of the ACTH-releasing activity. The hydrolysate also contains a potent histamine-like substance the activity of which, unlike that of P, is readily abolished by pyribenzamine. The releasing factor, provisionally designated as P_1, is apparently identical with the ACTH-releasing agent known to occur in commercial pitressin.

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Note on Calcium Ion Requirements for Threone Activity.* (22555)

SHIRLEY A. JOHNSON AND WALTER H. SEEGERS.

Department of Physiology and Pharmacology, Wayne University College of Medicine, Detroit, Mich.

The conversion of prothrombin to thrombin occurs in several ways. Two possibilities may be represented by the following equations:

$$\text{Prothrombin} \xrightarrow[\text{Ac-globulin, Thromboplastin}]{Ca^{++}} \text{Thrombin}$$

$$\text{Prothrombin} \xrightarrow[\text{Ac-globulin, Threone}]{Ca^{++}} \text{Thrombin}$$

In the first type of activation, thromboplastin of tissue origin is involved and it is known that a certain optimum calcium concentration best supports the reaction(s). This optimum is found to be about the same irrespective of whether whole blood or plasma is clotting (1,2) or whether purified prothrombin is being activated(3).

In the type of activation represented by the second equation, platelets are involved instead of thromboplastin; for, threone activity consists of the simultaneous presence, in suitable proportions, of platelet cofactor I from the plasma and platelet factor 3 from the platelets(4). This may be represented as follows: Platelet factor 3 + Platelet Cofactor I = Threone. In the present study we measured the calcium ion requirements for threone activity.

Methods. Purified prothrombin was prepared from bovine sources(5,6,7) and assayed by a modification of the 2-stage technic(8), and thrombin activity was measured as described by Seegers and Smith(9). Platelet suspensions were prepared as described previously(10); and in this study, it was found that they could also be replaced with purified platelet factor 3 obtained and assayed quantitatively for its activity as described(11). Platelet cofactor I concentrates were obtained from bovine sources and assayed according to procedures we hope to describe in detail in a later communication. Our preparations of platelet cofactor I were free of autoprothrombin II activity. The following reaction mixture was composed: (a) Purified prothrombin, 2400 u/ml; (b) platelet homogenates or purified platelet factor 3, 75 u/ml; (c) Platelet cofactor I concentrate, 65 u/ml; and, (d) $CaCl_2$ dissolved in imidazole buffer pH 7.25. The latter was the only variable. The reaction mixture was placed in a water bath at 28°C, and periodically samples were removed for the quantitative determination of thrombin activity. The results are presented on Fig. 1.

Results. In our study of the calcium ion requirements for the conversion of prothrombin to thrombin with threone, we found that there is no sharp optimum concentration. Once a certain minimum calcium ion concentration is supplied, the rate of prothrombin activation and the yield of thrombin is the same from 0.009 M to 0.04 M concentration (Fig. 1). With higher concentration, calcium is inhibitory. The wide range of calcium concentration most suitable for threone

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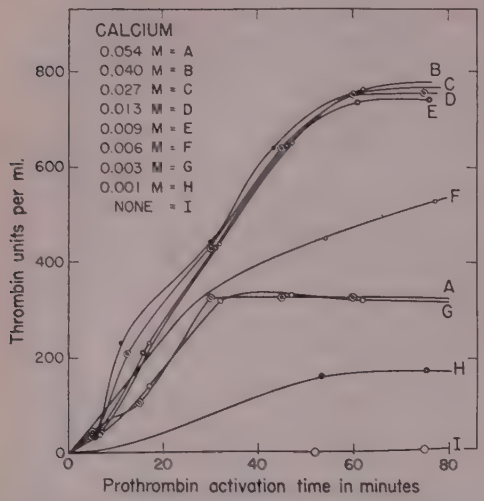

FIG. 1. Activation of purified prothrombin. Only calcium concentration was varied, as indicated for each curve. Molar concentration refers to concentration in reaction mixture described in the text.

activity is thus seen in sharp contrast with the narrow optimum range found with thromboplastin(3) as an activator of prothrombin.

Summary. When threone is involved in the conversion of purified prothrombin to thrombin, the optimum calcium concentration ranges from 0.009 M to 0.04 M. This is a much broader range than found when thromboplastin is used for the activation of prothrombin.

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Attempts to Identify Site of Production of Circulating 'Erythropoietin'. (22556)

ALBERT S. GORDON, SAM J. PILIERO, PAUL T. MEDICI, CHARLES D. SIEGEL, AND MYRON TANNENBAUM.

Departments of Biology, New York University and St. John's University, and Department of Anatomy, New York Medical College.

It is becoming rapidly established that humoral mechanisms underlie the erythropoietic effects of anoxia. Thus serum and plasma, or extracts derived therefrom, obtained from animals rendered anemic by bleedings(1-9), or by repeated injections of phenylhydrazine (9-13) exert considerable erythropoietic activity. Attempts to concentrate the active principle(s) from 'anemic' plasma and serum have led some to the conclusion that it is heat-stable and protein-free(9-15). In this connection, boiled filtrates of acidified plasma obtained from blood of phenylhydrazinized rabbits display marked erythropoietic actions in the rat (9-13). Relatively few investigators have attempted to locate the site of formation of the blood erythropoietic factor. Gordon *et al.*(5) detected slight activity in the livers of chronically bled rabbits and other experiments(16) appear to rule out those organs, presumably blood-forming in nature, which are injured by nitrogen mustard.

It seemed important to consider this problem in greater detail by subjecting a variety of organs to the chemical procedure employed

for obtaining 'erythropoietin' from plasma (10,11).

Materials and methods. Donor rabbits. Eight male New Zealand rabbits weighing 4-6 kg were injected daily for 2 days with 1 ml of a 2.5% solution of phenylhydrazine HCl (25 mg) neutralized to pH 7.1. On the 3d day and for the subsequent 2 days, they received 1.5-2.5 ml of this solution (37.5-62.5 mg), graded according to body weight. No treatment was administered on the 6th day because of the death of 2 animals. The remaining 6 animals were intensely anemic on the 7th day, displaying red cell counts of approximately one mill/mm^3 and reticulocyte values ranging from 82 to 96%. At this time, blood obtained from these rabbits by cardiac puncture was heparinized (10 mg/50 ml blood) and centrifuged for 15 minutes. The pooled plasmas (348 ml) were brought to a pH of 5.5 by the addition of 9.9 ml of 1 N HCl and boiled for 10 minutes. Filtration yielded 110 ml of extract. The livers, spleens, thymuses, lungs, brains (excluding medullae and pituitaries), gastrocnemii, bone marrows (femoral, tibial and humeral) and packed blood cells (82-96% reticulocytes) were collected for further preparation. The livers (604 g) were homogenized for 20 minutes in a Waring blendor; 100 ml of acid saline (pH 5.5) were added and the material was acidified to a pH of 5.5 by the addition of 12.1 ml of 1 N HCl. After further homogenization, 300 ml of acid water were added and the mixture was boiled for 10 minutes. Filtration followed the boiling at which time a total volume of 300 ml was collected. One hundred and twenty-five ml of acid saline were added to the splenic substance (25.5 g) during a 20-minute homogenization period. The material was then adjusted to a pH of 5.5 by the addition of 0.8 ml of 1 N HCl. The mixture was boiled for 10 minutes after which it was filtered. A volume of 95 ml of extract was obtained. Thymus material (32.5 g) was also homogenized during which period 125 ml of acid saline were added. The mixture was acidified further to pH 5.5 by addition of 0.21 ml of 1 N HCl. Boiling followed for 10 minutes after which time the material was filtered and 64.5 ml of filtrate collected. One hundred ml of acid saline were added to the lungs (77.5 g) during homogenization. The mixture was then acidified to pH 5.5 by addition of 1.37 ml of 1 N HCl. The material was boiled for 10 minutes and filtration yielded 103.5 ml of extract. The brain substance was homogenized (64.5 g) during which time 125 ml of acid saline were added. Further acidification to pH 5.5 was achieved by the addition of 0.37 ml of 1 N HCl. Boiling for 10 minutes was followed by filtration which yielded 74 ml of extract. One hundred and seventy-five ml of acid saline were added to the muscle tissue (369 g) during homogenization. The homogenate was acidified further to pH 5.5 by the addition of 1.72 ml of 1 N HCl and boiled for 10 minutes following which filtration yielded 188 ml of extract. Bone marrow tissue (60.3 g) was homogenized during which time 150 ml of acid saline were added. Following further homogenization, the mixture was acidified to pH 5.5 by addition of 2.32 ml of 1 N HCl. Boiling followed for 10 minutes after which filtration yielded 173 ml of extract. The packed blood cells (186 ml) were acidified to pH 5.5 by addition of 5.02 ml of 1 N HCl. This mixture was boiled for 10 minutes and filtered; 150 ml of acid saline were added to the residue which was then reboiled for 2 minutes. Filtration followed and both filtrates were combined resulting in a yield of 122 ml. All extracts were stored in a refrigerator at 5°C when not used and brought to room temperature just before injection.

Recipient rats. Nine groups, each consisting of 5 young adult female rats of a modified Long-Evans strain weighing 153-196 g, were employed to assess the hemopoietic activities of the 9 extracts. They were given daily subcutaneous injections for 10 days of the following volumes of extract: plasma—1 ml, equivalent to 2.9 ml of original whole plasma; liver—3 ml, equivalent to 6 g tissue; spleen—1.5 ml, equivalent to 0.39 g tissue, for the 1st 5 days followed by 5 daily 2 ml injections; thymus—1.2 ml, equivalent to 0.6 g tissue; lung—2 ml, equivalent to 1.4 g tissue; brain—1.3 ml, equivalent to 1.04 g tissue; muscle—3 ml, equivalent to 5.7 g tissue; bone marrow—3 ml, equivalent to 1 g

TABLE I. Effects of Boiled Filtrates of Blood and Organs upon Peripheral Red Cell Values in Rats (Means ± Stand. Errors).

	Body wt (g)	RBC (mill/mm³)	Hemoglobin (g/100 ml)	Hematocrit (%)	Reticulocytes (%)
Plasma					
Before	175.4 ± 5.9	7.98 ± .29	17.36 ± .70	50.1 ± 1.54	2.20 ± .23
After	184.2 ± 6.3	10.38 ± .45†	19.03 ± .61	58.0 ± 1.36†	4.40 ± .33†
Liver					
Before	165.2 ± 3.2	7.70 ± .25	16.95 ± .64	53.0 ± 2.28	3.22 ±1.71
After	170.8 ± 3.5	7.42 ± .12	16.83 ± .36	51.3 ± .97	2.30 ± .55
Spleen					
Before	166.8 ± 5.1	8.27 ± .15	16.84 ± .22	51.2 ± .73	2.80 ± .61
After	172.4 ± 5.6	7.74 ± .12*	16.34 ± .20	48.5 ± 1.24	2.80 ± .65
Thymus					
Before	173.6 ± 3.9	7.64 ± .15	16.04 ± .55	49.4 ± 1.58	3.60 ± .50
After	185.2 ± 5.5	6.92 ± .16*	15.59 ± .67	47.9 ± 1.47	2.50 ± .40
Lung					
Before	174.6 ±10.1	7.68 ± .26	16.38 ± .34	50.4 ± 1.39	1.70 ± .66
After	184.0 ± 8.7	7.74 ± .25	16.44 ± .38	47.9 ± 1.29	2.30 ± .26
Brain					
Before	153.2 ± 8.0	8.06 ± .25	16.62 ± .41	51.9 ± .65	2.30 ± .33
After	156.2 ± 6.8	8.07 ± .19	16.56 ± .18	50.7 ± 1.03	2.50 ± .08
Muscle					
Before	169.2 ± 4.8	8.24 ± .25	17.39 ± .42	52.2 ± 1.18	2.40 ± .25
After	172.4 ± 4.7	7.76 ± .08	16.83 ± .65	51.0 ± .98	1.90 ± .19
Bone marrow					
Before	175.6 ± 6.7	8.02 ± .17	17.33 ± .42	53.6 ± .54	1.76 ± .17
After	180.0 ± 7.2	8.54 ± .14*	17.39 ± .30	54.0 ± 1.41	2.04 ± .33
Packed red cells					
Before	179.4 ± 3.6	8.02 ± .27	16.07 ± .29	53.9 ± 1.98	3.20 ± .34
After	179.6 ± 3.1	7.74 ± .16	16.52 ± .70	55.5 ± 1.87	2.30 ± .45

* Indicates probability value, $P<0.05$ for the means 'after' as compared to those 'before' treatment.

† Indicates probability value, $P<0.01$.

tissue and packed cells—2 ml, equivalent to 3 ml of cells. All rats were sacrificed on the 11th day for determinations of the peripheral and bone marrow cellular values. Peripheral cell counts were also taken in all rats before experimental treatment was initiated. Forty untreated rats, studied over the course of a year, which overlapped the period of the present experiments, contributed to the control myelogram values. Freely flowing blood from the tail was employed for the peripheral cell determinations. Red cell counts were made in duplicate and differential counts were determined from smears stained with Wright's. Eosinophil numbers were estimated by the chamber method using Randolph's fluid(17). Reticulocyte counts were made from dry smears of heparinized blood stained with new methylene blue(18); 1000 red cells were counted for each determination and the reticulocytes expressed as a percentage of these. Hemoglobin concentrations were estimated by the acid hematin method with a photoelectric colorimeter. Duplicate hematocrit values from heparinized blood, spun at 11,000 rpm for 5 minutes, were obtained by a capillary micromethod. At termination of the experiments, all recipient rats were anesthetized with ether and exsanguinated by cardiac puncture. The right femur was dissected, split lengthwise and the bone marrow removed and placed in a watch glass containing homologous serum. The marrow was prepared in suspension form by gently drawing it up and down in a glass pipette. Smears were then made, fixed immediately in absolute methanol for 2-5 minutes and treated with May-Grünwald stain. For each animal, a minimum of 1500 nucleated cells was counted and classified according to previously reported procedures(19).

Results. Table I indicates that the acidi-

TABLE II. Effects of Boiled Filtrates of Blood and Organs upon Myelograms of Rats (Mean % ± Stand. Error).

	Nuc. RBC	Lympho-cytes	Neutrophils* Young	Neutrophils* Mature	Eosinophils Young	Eosinophils Mature	Blasts	Misc.
Untreated controls	35.1 ± 1.9	8.9 ± 2.0	9.0 ± .7	36.8 ± 1.0	.2 ± .5	7.6 ± 1.0	.2 ± .8	2.2 ± .8
Plasma	51.8 ± 3.7‡	8.6 ± 3.4	7.7 ± 1.6	25.4 ± 2.6‡	.3 ± .2	4.7 ± 1.0	None	1.5 ± .4
Liver	33.1 ± 3.5	6.0 ± 1.3	15.3 ± 2.4†	35.8 ± 1.5	.8 ± .6	6.6 ± 1.7	.2 ± .2	3.2 ± .9
Spleen	29.9 ± 3.2	6.8 ± 2.7	16.0 ± 1.8‡	31.0 ± 3.6	1.2 ± .8	12.8 ± 2.2†	.3 ± .2	4.0 ± .5
Thymus	35.6 ± 2.4	5.2 ± 1.4	11.6 ± .7	32.4 ± 3.1	.2 ± .2	7.6 ± 1.2	None	5.6 ± 1.0†
Lung	29.6 ± 3.7	7.4 ± 1.7	11.4 ± 1.8	38.4 ± 1.5	1.0 ± .2	9.6 ± 1.3	"	3.2 ± 1.4
Brain	38.4 ± 3.7	5.2 ± 1.8	12.6 ± 2.5	37.0 ± 3.8	.4 ± .4	5.0 ± .9	.4 ± .2	1.0 ± .4
Muscle	33.8 ± 3.3	9.6 ± 2.2	9.4 ± 2.6	38.2 ± 1.2	.4 ± .2	6.0 ± .9	None	2.6 ± .7
Bone marrow	31.0 ± 5.2	6.8 ± 2.4	13.4 ± 2.3	41.4 ± 6.8	None	5.2 ± 1.3	.2 ± .2	2.0 ± .2
Packed red cells	39.0 ± 2.0	5.0 ± 1.6	16.4 ± 2.7†	34.0 ± 2.0	.2 ± .2	3.8 ± 2.0	.2 ± .2	1.4 ± .7

* Young neutrophils include promyelocytes and myelocytes; mature neutrophils include segmented forms.

† Indicates probability values, $P<0.05$ for means compared to controls.

‡ " " " , $P<0.01$.

fied boiled filtrate of plasma obtained from phenylhydrazinized rabbits resulted in highly significant rises in the peripheral red cell, hematocrit and reticulocyte values as well as a trend towards an increase in hemoglobin levels in the recipient rats. The elevation in red cell numbers exceeded, percentagewise, that experienced by the hematocrit and hemoglobin values. Apart from a slight increase in the red cell counts brought about by the marrow extract and slight decreases induced by the spleen and thymus extracts, none of the organ or packed cell filtrates evoked any significant changes in the peripheral red cell parameters. Substantial body weight gains were experienced by most groups except those injected with packed cells, muscle and brain material.

Total white, differential and eosinophilic cell (chamber count) values were not altered significantly by the filtrates of plasma, packed cells or any of the organs employed.

Table II shows that among the different materials tested, only the plasma extract stimulated erythropoiesis within the femoral bone marrow. The highly significant increases in the percentages of nucleated erythrocytes, induced by the plasma filtrate, were accompanied by a drop in the percentages of mature neutrophilic forms. The extracts of liver, spleen and packed cells induced increases in the percentages of young neutrophilic elements while those of spleen and thymus evoked rises in the percentages of mature eosinophilic cells and miscellaneous elements respectively. No other significant actions were exerted by the tissue extracts upon the marrow parameters examined.

Discussion. The present results confirm previous reports(5,9-13) that boiled filtrates of plasma obtained from anemic rabbits contain considerable erythropoietic activity. This is evidenced by the significant elevations in peripheral red cell numbers, hematocrit and reticulocyte percentages and by the increased percentages of marrow nucleated erythrocytes in the rats receiving daily injections of this filtrate. It is of interest that in these experiments, unlike those previously reported (10,13), the percentage increases in peripheral red cell numbers were greater than those displayed by the hematocrit and hemoglobin values, which is indicative of the production, in response to the filtrate, of smaller-size erythrocytes associated with a lowered corpuscular hemoglobin. Similar results have been reported by Linman and Bethell(9). The explanation for the microcytosis is not evident although preliminary experiments(20) tend to indicate that alterations in iron metabolism may be responsible for this phenomenon.

None of the organ or packed cell extracts employed in this study proved to be erythropoietic. This should not be considered as indicating necessarily that the various tissues examined do not participate in the production of the erythropoietic factor. It is conceivable, for example, that an efficient storage

mechanism for this principle does not exist, with the factor being released into the circulation as rapidly as it is produced. The present results indicate that plasma may be the only site from which appreciable amounts of 'erythropoietin' are recovered. Experiments are in progress to determine whether the factor may actually be formed in plasma as the result of an enzymatic action upon substrates provided by the blood-forming organs.

Summary. Acidified boiled filtrates were prepared of plasma, liver, spleen, thymus, lung, brain, skeletal muscle, bone marrow and packed blood cells of rabbits made severely anemic by phenylhydrazine. When tested in normal rats, only the plasma extracts proved to be erythropoietic, as evidenced by significant elevations in peripheral red cell, hematocrit and reticulocyte values as well as by a marked increase in the percentages of marrow nucleated erythrocytes. The site of formation of circulating 'erythropoietin' remains unidentified.

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Canine Intestinal and Liver Weight Changes Induced by *E. coli* Endotoxin. (22557)

L. D. MACLEAN, M. H. WEIL, W. W. SPINK, AND M. B. VISSCHER.
Departments of Physiology, Surgery and Medicine, University of Minnesota Medical School, Minneapolis.

Shock has been observed in the human following transfusion of contaminated blood(1), the injection of purified products of bacteria (endotoxin) to induce hemorrhage in malignant tumors(2), and following the treatment of Gram-negative bacteremia with antibiotics. Irreversible hemorrhagic shock in the dog has also been related to the deleterious effects of bacterial products(3). Previous investigation(4) has shown that the intravenous injection of endotoxin prepared from *Escherichia coli* regularly results in a profound decline in systemic blood pressure with an accompanying elevation of portal vein pressure. This prompt hemodynamic reaction did not occur in the hepatectomized animal. Three possible explanations for this phenomenon are evident: 1) large quantities of blood are trapped in the liver, 2) the liver releases vasodepressor substances or 3) both 1 and 2 occur. Attempts

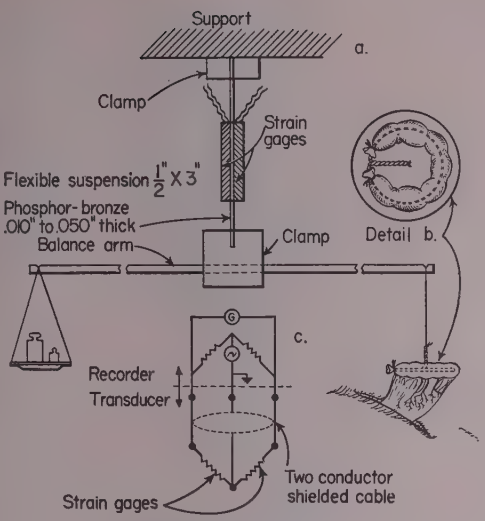

FIG. 1. Diagrammatic representation of weighing device including electrical circuit. See text.

to quantitate the volume changes of the liver following endotoxin administration by means of a plethysmograph were unsuccessful and led to the use of the method herein described. It has also been observed(4) that following the precipitous decline in blood pressure following intravenous endotoxin administration in the dog, a partial recovery occurs, coincident with return of portal vein pressure to normal, followed subsequently by a second decline in blood pressure leading to death. An estimation of pooling of blood in viscera other than the liver to account for the secondary hypotension was considered necessary. The small intestine was chosen and these results are included with a comparison of weight changes in the liver.

Methods. 1) Device for Continuously Recording Changes in Weight of Suspended Organs *in vivo*. The instrument illustrated in Fig. 1 has proved practical in estimating weight changes in organs *in vivo*. The performance characteristics demonstrate stability, sensitivity, and linearity adequate for the present physiological study(5). The weighing device consists of a rigid support and clamp from which is suspended a phosphor-bronze strip ½" x 3" and .01" to .05" thick, which in turn supports a balance arm 29" in length, at its midposition. There is provision for suspension of an organ to be studied at one end of balance arm and counterpoise at the opposite end. Strain gauge elements are cemented to each side of the phosphor-bronze strip so that any deflection of the balance arm will result in an increase in resistance in one strain element and a decrease in resistance in the opposite strain element; hence, an alteration in potential as measured by galvanometer G illustrated in C, which also includes a diagram of the resistances to electrical flow incorporated in strain gauges and in recorder. Differences in liver weight of 5 g and differences in small intestinal loop weight of one gram could be accurately detected. (2) Suspension of the Liver. The liver was exposed through a midline abdominal and sternotomy incision and mobilized by dividing the right and left triangular ligaments of the liver. The diaphragm was divided from the midline anteriorly to the inferior vena cava immediately cephalad to the liver. The right, left and quadrate lobes of the liver were elevated and enveloped in a net, the mouth of which was closed about the liver tightly enough for support only. The portal vein pressure was measured from a tributary of the splenic vein. All experiments were performed with a suspension which did not elevate portal vein pressure. All animals had a normal blood pressure at the beginning of the experiment suggesting that inferior vena cava flow was not compromised. The temperature of the liver was never less than 35°C. (3) Suspension of Intestine. A segment of small intestine from 25 to 60 cm in length with intact blood and nerve supply was isolated from the remaining bowel. Covered wire 0.5 cm in diameter was passed through the lumen of the isolated segment of bowel and utilized for support without tension on the mesentery and avoiding contact with other organs, (Fig. 1B). The loop was moistened with physiological saline at 37°C. During control observation periods of over one hour, the intestinal loops decreased in weight at the rate of one to 2 g per 25 cm per hour. All blood pressure records were made using strain gauges with optimal damping, recorded simultaneously and continuously with liver or intestinal

TABLE I. Changes in Liver and Intestinal Weight in the Dog following Systemic Injection of *Escherichia coli* Endotoxin.

Dog wt, kg	Type and dose of endotoxin	Min. from inj. to max incr. in liver wt	Max incr. in liver wt, g	Min. from ins. to return to normal liver wt	Simultaneous decline in mean systemic blood pressure, mm/Hg	Simultaneous incr. in portal vein pressure, mm/Hg	Min. from inj. to max incr. in intestinal wt and B.P. at that time	Max incr. in wt of intestinal loop, g/cm	Estimated storage of fluid in entire S. intestine, ml	Estimated storage of fluid in entire S. intest. at height of portal pressure rise, ml*
12	"Crude," .3 ml/kg	3	80	5.6	40	9	60 B.P. 25/0	18	770	55
19	"	1.5	350	25	100	19	50 B.P. 60/40	.8	340	170
12	"	1.5	90	5	35	15	50 B.P. 75/50	.5	210	56
15	"	1	80	5	50	19	55 B.P. 115/60	.5	196	77
13	Purified, 5 mg/kg	3	100	7	50	12	75 B.P. 110/80	.2	70	46
10	"	—	—	—	—	—	55 B.P. 100/65	.3	98	28

* Due to back pressure from the liver occurring at time of max increase in liver wt.

weight on a Sanborn Polyviso recorder. All animals were adult mongrel dogs. Anesthesia used was sodium pentobarbital 30 mg/kg. The preparation of endotoxin has been previously described(4).

Results. 1. *Changes in liver weight of dog due to* E. coli *endotoxin.* The results are illustrated in Table I, and Fig. 2. An increase in liver weight occurs within 3 minutes of endotoxin injection which is accompanied by a rise in portal vein pressure, and fall in systemic arterial blood pressure. The liver returns to normal weight in from 5 to 25 minutes during which time there is a return of arterial blood pressure in the direction of normal in most animals. While the quantity of blood trapped in the liver is enough to account for the decline in blood pressure observed in most experiments the possibility exists that the liver is producing a vasodepressor substance which contributes to the initial hypotension.

2. *Changes in small intestinal weight of the dog due to* E. coli *endotoxin.* The results are illustrated in Table I and Fig. 2. The storage of fluid in the small intestine due to back pressure from liver which occurs during the initial shock phase is in most experiments minor. A later large gain in intestinal weight occurred regularly at a time when the liver weight and portal pressure had returned to normal. Those animals with the lowest blood pressure at the time of maximal intestinal weight demonstrated the greatest increase in intestinal weight. The liver weight at this time (50 to 75 minutes) following endotoxin injection was at or below the preinjection level.

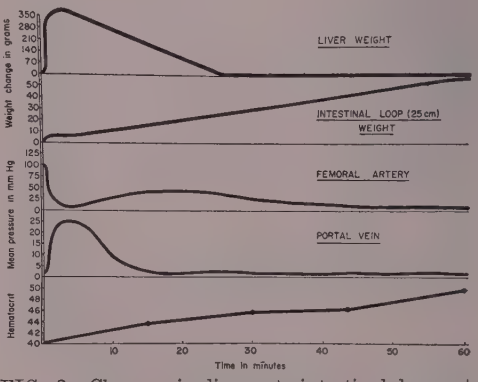

FIG. 2. Changes in liver wt, intestinal loop wt, femoral artery pressure, portal vein pressure and hematocrit for first hour following systemic *E. coli* endotoxin injection. Liver wt increased 350 g in a 19 kg animal within 1.5 min. A simultaneous increase in portal vein pressure and decline in femoral artery pressure occurred. A small elevation of intestinal loop (25 cm) weight occurred at this time, but a much larger rise in intestine weight occurred later, when liver weight had returned to normal. The latter rise is accompanied by a second decline in systemic blood pressure.

Discussion. There is some doubt that the component of the hypotension due to liver congestion is not species specific to the dog We have noted the same reaction as described for the dog in the rabbit and rat suggesting that this is not a species specific reaction. Since an elevation of portal vein pressure and hypotension can be produced with histamine, a bio-assay for this material was performed on the endotoxin utilized in this investigation. The "Crude" endotoxin contains between 0.5 and 1.5 μg of histamine per ml which is equivalent to a maximum of 10 μg in the largest dog studied and is not of significance in contributing to the marked weight gain observed in the liver or intestine. The purified endotoxin, Shear's endotoxin and crude endotoxin prepared from synthetic media do not contain histamine but give indistinguishable results.

A similar reaction to that described due to endotoxin (decline of systemic arterial pressure and elevation of portal vein pressure) has been noted following the systemic injection of certain peptone solutions of high protease content(6). While these reactions might be related, the shock, elevated portal vein pressure and increase in liver weight have been observed with the highly purified endotoxin of Shear and endotoxin grown on synthetic media both of which give a negative Biuret test for the peptide linkage(5). The site of obstruction leading to increase in liver weight is believed to be on the hepatic venous side of the liver sinusoids because microscopic examination of the liver reveals central vein congestion and vacuolization about the central vein. Additionally, when the isolated, weighed liver is perfused through the portal vein and hepatic artery using a constant flow pump, the addition of endotoxin results in increase in weight, an elevation of portal vein pressure, hepatic artery pressure and a decreased outflow during the period of weight increase. An increase in intestinal loop weight occurred at a time when the liver weight had returned to normal. The hematocrit invariably increases in these animals at this time, (Table I). The increase in small intestinal weight is believed to be due in considerable part to pooled or extravasated blood although fluid storage as edema is not excluded. The intestine is grossly hemorrhagic. This organ would appear to be the site of a vascular response induced by endotoxin possibly on a basis of previous sensitization.

Summary. 1. Systemic arterial blood pressure response of the dog to injection of *Escherichia coli* endotoxin is characterized by a sudden precipitous drop, a partial recovery and a subsequent slower decline over a period of hours. 2. Utilizing a device for quantitatively estimating changes in weight of organs *in vivo,* an increase in liver weight of from 80 to 350 g has been noted within one to 2 minutes following injection of *E. coli* endotoxin. 3. Simultaneous increase in weight of small intestine of lesser magnitude (28 to 170 g) was noted at the same time and is believed to be due to back pressure from the liver. Increase in liver and intestinal weight were believed to be quantitatively sufficient to account for the hypotension observed. 4. A difference in reaction of the liver and intestine was noted. Whereas the liver returns to normal weight within 5 to 25 minutes, the small intestine continues to increase in weight reaching a maximum of from 196 to 770 g approximately one hour following endotoxin injection. 5. The secondary hypotension due to endotoxin is believed to be at least partially the result of loss of blood in the small intestine due to vascular damage in this organ.

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Antigens of Human Seminal Plasma.* (22558)

Alfred J. Weil, Olga Kotsevalov and Leo Wilson.
Departments of Bacteriology and Gynecology, The Bronx Hospital, New York 56, N. Y.

The spermatozoa of man and other animals contain antigens of marked specificity(1-4). However, human seminal plasma has not been examined by immunological technics.† As to animals, Smith(4) produced one antiserum against rabbit seminal plasma in a goat, which reacted with seminal plasma, but did not agglutinate rabbit spermatozoa, whereas antispermatozoal sera did crossreact with spermal plasma. The immunological properties of human seminal plasma and the peculiar relations of the antigens of seminal plasma to those of human spermatozoa are the subject of this report.

Technics. Human semen specimens were obtained from private and clinic patients.‡ Except for agglutination tests necessitating the use of motile spermatozoa, specimens could be preserved in the refrigerator for several weeks without noticeable impairment of immunological activity. Dilutions were made in 0.85% NaCl solution, except where otherwise noted. Separation of seminal fluid and spermatozoa was done in a high speed angle centrifuge (Lourdes) by spinning for 10 min. at 10,000 rpm. The sediment was washed by repeated centrifugation with saline, until no antigen could be detected in the supernatant fluid by complement fixation test. The spermatozoa thus collected always contained small amounts of cellular (epithelial?) detritus and a few leukocytes. For sonic disintegration a Raytheon Magnetostriction Oscillator (9 kilocycles, 50 W.) was used. Spermagglutination tests were made according to the method outlined in(6). Precipitation tests were made by layering undiluted immune serum beneath varying dilutions of antigen. After reading for ring formation, the tubes were shaken and further readings were made after incubation for 2 h at 37°C and finally after standing in the refrigerator overnight. Tests with the agar diffusion method utilized the procedure outlined by Vaughan and Kabat(7). Complement fixation tests were performed according to the standard Kolmer method with 2 "full" units of complement. Immune sera were produced in rabbits either by a series of intravenous injections, or by 3 intramuscular injections of equal volumes of antigen and Freund's adjuvant.§ No difference in the behavior of the antisera obtained by these 2 methods was noted. Roosters were immunized by a series of intravenous injections.

Serum specimens obtained before immunization and sera of other untreated animals were used as controls. As no reactivity was seen in any of our tests with these controls or with the antigens used, they are not mentioned further in the text or the tables. Agglutination tests with fresh or tannic acid treated erythrocytes for adsorption of antigen were made according to the method given in (8).

Observations. Regardless of whether whole semen, seminal plasma or washed spermatozoa were injected, the rabbit immune sera, in serum dilutions individually varying between 1/80 and 1/640, reacted in complement fixation tests with seminal plasma in dilutions up to 1/100,000 or slightly more. This is exemplified in Tables I and II. The same sera also reacted with washed spermatozoa. One to four million spermatozoa (in 0.1 ml saline solution) sufficed to yield complete inhibition of hemolysis with 0.01 to 0.0006 ml immune serum. Rooster immune sera did not give complement fixation.

Both rabbit and rooster immune sera pre-

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† Ross *et al.*(5) mention preparation of immune serum against seminal plasma, but data on work done with it have not been found.

‡ The assistance of Dr. R. Kurzrok in providing numerous semen specimens is gratefully acknowledged.

§ Generously provided by Mr. A. Lane of Difco Laboratories.

TABLE I. Fixation of Complement by Rabbit Immune-Serum against Whole Human Semen (Diluted 1/20) and

Antigen dilution	a Whole semen 1/1000	b Seminal plasma (supernate from a) 1/1000	c Spermatozoa (6×10^6/0.1 ml)	d Last washing fluid from c 1/5
1/ 1	4+	4+	4+	—
1/ 2	4+	4+	4+	—
1/ 4	4+	4+	—	—
1/ 8	4+	4+	—	—
1/ 16	4+	4+	—	—
1/ 32	4+	4+	—	—
1/ 64	2+	2+	—	—
1/128	—	—	—	—

In this and the following tables, degree of hemolysis is represented as follows: 4+, no hemolysis; 3+, slight (approximately 30%); 2+, moderate (approximately 50%); 1+, strong (approximately 70%); (+), almost complete; —, complete hemolysis.

TABLE II. Fixation of Complement by Rabbit Immune Sera (Diluted 1/10) and

	Seminal fluid 1/1000		Washed spermatozoa 1×10^6/0.1 ml	
Antigen dilution	a Anti-seminal plasma serum	b Anti-spermato-zoal serum	c Anti-seminal plasma serum	d Anti-spermato-zoal serum
1/ 1	4+	4+	4+	4+
1/ 2	4+	4+	4+	4+
1/ 4	4+	4+	4+	4+
1/ 8	4+	4+	4+	4+
1/ 16	4+	4+	(+)	—
1/ 32	4+	4+	—	—
1/ 64	4+	4+	—	—
1/128	—	—	—	—

cipitated seminal plasma in dilutions up to 1/1000 or 1/10,000. Precipitation by the rooster sera was slower than with rabbit sera. Their reactions were not accelerated or increased by raising the NaCl content of the system to 4 or 8%.

Precipitation of seminal plasma was also obtained by the agar diffusion method with all the sera from both animal species. The zones observed with rooster sera were less clear cut than those with rabbit sera. With the latter, 3 regions of precipitation could usually be seen, namely a zone of heavy precipitation which moved with extreme slowness; a zone of precipitation moving with greater speed and often subdivided in many (up to 10) bands located very close to each other; and finally a faint band moving more rapidly than the second one. Individual specimens of seminal plasma show a considerable variation in the number of bands produced and in the speed with which they move with the same immune serum. This handicapped the identification of bands considerably. No consistent differences could be ascertained between the behavior of sera against seminal plasma and of those against spermatozoa.

Pre-treatment and other control rabbit or rooster sera agglutinated equally fresh and tannic-acid treated human erythrocytes (Group O) in dilutions between 1/5 and 1/20. (Rooster serum may also cause partial hemolysis in dilutions of this order.) Treatment of fresh erythrocytes with seminal plasma did not result in agglutination with immune sera beyond that seen with the controls. However, erythrocytes treated with tannic acid and then brought in contact with seminal plasma were agglutinated by our immune sera in dilutions of 1/10,000 or better. Again, there was no significant difference in the reactions of anti-seminal plasma and anti-spermatozoa immune sera.

TABLE III. Fixation of Complement by Rabbit Immune Sera (Diluted 1/10) and Antigens (Human).

Antigen dilution	A Seminal plasma 1/1000			B Serum 1/100			C Kidney 1/5			D Liver 1/5			E Spleen 1/5			F Atrophic testis 1/5		
	Anti-seminal plasma	Anti-spermatozoa	Anti-serum	Anti-seminal plasma	Anti-spermatozoa	Anti-serum	Anti-seminal plasma	Anti-spermatozoa	Anti-serum	Anti-seminal plasma	Anti-spermatozoa	Anti-serum	Anti-seminal plasma	Anti-spermatozoa	Anti-serum	Anti-seminal plasma	Anti-spermatozoa	Anti-serum
1/ 1	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
1/ 3	4+	4+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
1/ 10	4+	4+	—	4+	3+	4+	4+	2+	4+	4+	2+	4+	4+	4+	4+	4+	4+	4+
1/ 30	4+	4+	—	2+	(+)	4+	—	—	4+	(+)	—	4+	—	—	4+	—	—	4+
1/ 100	4+	4+	—	—	—	4+	—	—	3+	—	—	4+	—	—	4+	—	—	4+
1/ 300	—	—	—	—	—	4+	—	—	—	—	—	2+	—	—	1+	—	—	4+
1/ 1000	not done			—	—	4+	not done			not done			not done					
1/ 3000				—	—	4+												
1/10,000				—	—	—												
1/30,000				—	—	—												

Attempts failed to remove cross-reacting antigen from the spermatozoa both by extended washing in saline and by treatment by sonic vibration. After 5 min. treatment in the oscillator, the spermatozoa had lost their tails. After more extended treatment—up to one hour—the number of spermatozoal heads collectible by centrifugation became very small (exact counts were impossible because of clumping), and the remaining heads were visibly deformed. This effect became particularly pronounced if sonic treatment was carried out in 8% rather than in 0.85% NaCl solution. However, the heads remained just as reactive in the complement fixation tests as were the original spermatozoa.

When rabbit antisera were absorbed with spermatozoa, the antibodies were completely removed. The matter of differential absorption needs further study.

We had occasion to examine the ejaculates of 3 persons which did not contain spermatozoa due either to obstruction of the vas deferens or to failure of spermatogenesis. These ejaculates were just as active in their reactions with our antisera as were the seminal fluids of normal individuals.

The cross-reactivity of our antisera with other materials of human origin was studied. About half of 15 sera did not react in precipitation and complement fixation tests with human sera. The others showed reactivity varying from traces to reactions at considerable dilutions, but never more than about 1/100 of the reactivity of antisera versus human serum. In agar diffusion tests, the sera nonreactive in complement fixation did not produce precipitation at all. The most cross-reactive sera showed one weak, slowly moving band and a very faint one moving slightly faster. Anti-human serum immune sera showed only very scanty cross-reactivity with seminal plasma, but the most reactive of them (which is the one shown in Table III) showed in agar precipitation 3 well defined bands with seminal plasma. Of these the middle one exhibited several sub-zones similar to the ones described for the homologous system.

Saline extracts from human organs were

prepared by suspending finely minced tissue freshly obtained from autopsies in 10 times their (wet) weight of saline solution containing 1/10,000 merthiolate, whirling for 5 min in a Waring blendor, and filtering through paper. These extracts could be used in 1/5 dilution in complement fixation tests without anticomplementary action. Extracts have been made from kidneys, livers, spleens, and from the atrophic testes of an 82-year-old man. Results of complement fixation tests with these extracts are exemplified in Table III, together with those with an anti-human serum immune serum. Cross reactivity as judged by these tests is slight. Similarly, precipitation tests with these extracts against anti-seminal plasma and anti-spermatozoal immune sera in fluid systems and by agar diffusion were entirely negative.

No information is as yet available concerning the chemical nature of the antigenic material in seminal plasma. Most of the *in vitro* activity is lost after 5 min. boiling undiluted or diluted 1/10 in saline solution. A small remainder stayed reactive even after 2 hours boiling. Only traces of material reactive *in vitro* could be extracted with organic solvents, such as alcohol, acetone, ether, or petroleum ether.

Two specimens of human serum containing the autoagglutinative antibody against spermatozoa described by one of us(6) were found not reactive in complement fixation tests with seminal fluid and with spermatozoa. No precipitation was observed in fluid systems with seminal plasma as antigen. However, in the agar diffusion test, the sera of both patients yielded with seminal plasma a weak and slowly moving zone, which was not seen if other human sera were employed.

Discussion. Seminal plasma contains highly antigenic material. It was impossible to distinguish by immunological methods between seminal plasma and spermatozoa with the technics employed. It remains uncertain whether the spermatozoa have antigenically active components of their own. A re-evaluation of the formerly collected data on the antigenic behavior of spermatozoa in the light of the evidence recorded here is indicated. It is likely that the antigenic material originates from the fluid products of the genital tract rather than from the spermatozoa because ejaculates free of spermatozoa show the same immunological behavior as does seminal plasma from normal semen. The observation that extracts from degenerated testes showed no more cross reactivity than other organs indicates also that the testis is not a major source of antigen. The testes from which the extract was prepared showed many immature germinal cells, though very few mature spermatozoa.

The data available make it likely that more than one antigenic component is present both in the seminal plasma and in or on the spermatozoa. The major portion of these antigens is of pronounced organ-specificity. It is remarkable that about half of our immune sera did not give any cross-reaction with human serum as antigen. A quantitative appraisal cannot, of course, be attempted as long as the general nature of the antigens involved remains unknown. Tests for species specificity have not yet been done. Arrangements are being made for collecting animal semen for this purpose. The data presently available on the species specificity of spermatozoa (1,2,4) would seem to be in need of supplementation by data on the immunological behavior of seminal plasma. The observations of Smith(4) suggest that what has been found with human semen must not be necessarily true for other animals.

The lack of reactivity of the sera with human spermagglutinating antibody in the sensitive complement fixation test is of interest. The agar diffusion test yielded a weak, but unmistakable zone of precipitation. This makes it likely that one of the antigenic components reactive with our immune sera is also related to the agglutinating antibody found in a few human beings.

Summary. Rabbits and roosters injected with whole human semen, seminal plasma and washed spermatozoa produced antisera which showed a high degree of specificity for seminal antigens as compared with human serum and organ extracts. However, a distinction be-

tween seminal plasma and spermatozoa could not be obtained with immunological technics. Evidence which would make it seem likely that the dominant antigenic material is derived from the fluid constituents of the semen rather than from the spermatozoa is presented. Human spermagglutinating sera do not give complement fixation tests with seminal plasma or spermatozoa, but they produce a zone of precipitation with seminal plasma as antigen in the agar diffusion test.

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Hemodynamic Effects of Vasopressor Agent (Metaraminol)* on Hypotension in Dogs Produced by Endotoxin. (22559)

MAX H. WEIL, LERNER B. HINSHAW,† MAURICE B. VISSCHER, WESLEY W. SPINK AND LLOYD D. MACLEAN.

Departments of Medicine, Physiology and Surgery, University of Minnesota Medical School, Minneapolis.

The mechanism of hypotension which occurs during the course of overwhelming infections has been studied intensively both in patients and experimentally in animals(1-5). The very striking hemodynamic changes which are sometimes observed in patients with bacteremia due to Gram-negative organisms have been attributed to the action of endotoxins which are liberated on dissolution of bacteria(6) and experimental shock may be readily produced by the administration of endotoxins derived from various Gram-negative bacteria. We had previously observed that intravenous injections of endotoxins, such as those obtained from *Escherichia coli* or *Brucella melitensis*, uniformly cause a prompt decline in arterial blood pressure in the anesthetized dog(7). This marked fall of blood pressure was shown to be the result of a corresponding fall in cardiac output without decrease in peripheral resistance. When total venous return was measured, the decreased cardiac output was found to be due to a marked deficiency in venous return(5,8). These changes were further correlated with a sharp increase in liver and intestinal weights (9). Our observations led to the tentative conclusion that the initial cardiovascular disturbances caused by various Gram-negative endotoxins in the dog are the end result of massive venous pooling in visceral organs(8).

The value of vasopressor agents in the treatment of shock has been controversial. Rashkind and co-workers(10) reported that norepinephrine normally increases venous return in the dog. Von Euler(11) expressed the opinion that norepinephrine has important vasoconstrictor effects on both arterial and venous vasculature thereby favoring effective circulation in patients with shock. Clinical experience with a related sympathomimetic amine, metaraminol (Aramine), has shown that this new vasopressor substance was effective in the management of shock associated with bacteremia(12). In an attempt to gain further insight into the influence of vasopressor agents on the course of shock, the effect of metaraminol on venous return in dogs was

* Metåraminol was supplied as Aramine by Medical Division, Sharp and Dohme, Division of Merck & Co., West Point, Pa.

† Life Insurance Medical Research Fellow.

TABLE I. Volume of Blood Pooled after Injection of Endotoxin into Dogs.

	No. of dogs	Time after inj. of endotoxin (min.)	Vol pooled, ml/kg Mean	Range
Controls	6	5	40.6	22- 61
		10	58	28- 82
		30	79.6	43-114
Animals receiving metaraminol	5	5	24.8	16- 31
		10	23.4	20- 31
		30	19.4	11- 31

studied after the administration of endotoxin.

Materials and methods. Observations were made following the intravenous injection of *E. coli* endotoxin of the Boivin type or a suspension of the dried bacterial cells which had been grown on synthetic media. The method for preparing the endotoxin has been described elsewhere(13). The dried bacterial cells, suspended in an equal volume of saline, were prepared according to a method described by Braude *et al.*(14). Previous experiments in intact animals had demonstrated that a profound fall in blood pressure and marked elevation of portal vein pressure regularly followed the intravenous injection of 5 mg/kg of purified endotoxin or 0.3 ml of bacterial suspension(5). This was the dosage used in the present experiments. Direct measurements of the venous return were made by a method more fully described elsewhere(5,8). Briefly, blood was diverted from the superior and inferior vena cavae to a cylindrical reservoir and pumped at a constant rate from the reservoir to the right atrium. The azygos vein had been ligated so that all blood returned to the heart was passed through the reservoir. The volume in the reservoir was continuously recorded. Because the amount of blood pumped from the reservoir to the right atrium was kept constant, changes in the reservoir volume were the result of quantitative changes in venous return. The system was initially filled with blood from a donor animal and measured quantities of blood were added when pooling in the animal caused depletion of the reservoir.

Adult mongrel dogs were used and all experiments were done under pentobarbital anesthesia in a dosage of 30 mg/kg. Arterial pressures were measured in the femoral artery. Pressures were recorded by means of Statham strain gauge manometers and a Sanborn polyviso recorder. Endotoxin was injected into the venous tubing leading to the right atrium. Purified *E. coli* endotoxin was used in 2 control and 3 treated dogs. The suspension of bacterial cells was employed for the remaining experiments. In this and earlier studies, the hemodynamic response to either preparation was found to be approximately the same.

Atropine sulfate in a dosage of approximately 0.5 mg/kg was given immediately after injection of endotoxin to 3 of 5 experimental animals. This agent alone had previously been shown to have no significant effect on venous return but it prevented arrhythmias of vagal origin produced by metaraminol which had invalidated several earlier experiments. Metaraminol in a dosage of 0.025 mg/kg was injected into the venous tubing after the sharp decline in venous return had clearly established itself. The initial dose was injected at approximately 3½ minutes (2-6 minutes) and supplementary doses were injected directly into the reservoir at 1½ to 8 minute intervals thereafter.

Results. The volume of blood pooled in the body of 6 control and 5 metaraminol-treated dogs is indicated in Table I. Results of these measurements are also illustrated in Fig. 1, where the heavy lines indicate the mean values and the shaded areas the spread of individual cases. It will be noted that following the injection of endotoxin, there was a rapid early loss of blood and a continued more gradual loss in the control series. The same pattern occurred initially in the test group. However, metaraminol promptly prevented further pooling and its effect is already apparent at 5 minutes. In the following 25 minutes small supplementary doses of metaraminol were added and the venous return was slightly increased indicating that some reversal of storage was occurring. The loss of blood due to pooling should be corrected for slight bleeding from the operative sites, but this correction would not minimize the differences shown.

Discussion. The initial circulatory dis-

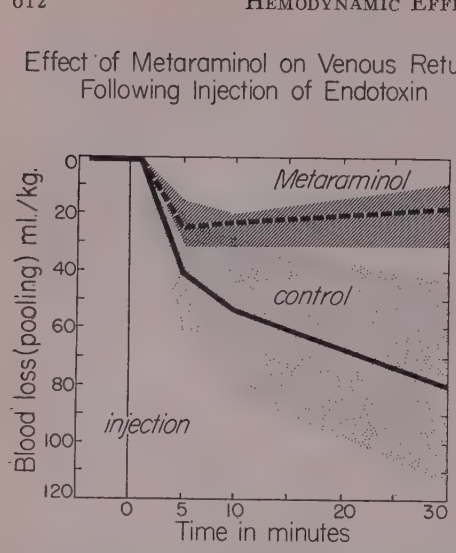

FIG. 1. Effect of metaraminol on venous return following inj. of endotoxin. Endotoxin was inj. at time zero. Metaraminol was inj. shortly thereafter when the sharp decline in venous return had begun.

turbance produced by endotoxin has been traced to the pooling of large quantities of blood in specific venous channels, particularly in the liver and intestine. The precise mechanism and location of this effect of endotoxin have not been investigated fully. It would appear that metaraminol has a selective effect on the venous vasculature which prevents and/or reverses pooling of blood in these vessels. Vasopressor agents may have a specific dilating action on hepatic veins and thereby counteract massive pooling in the splanchnic bed brought about by their constriction(15). The use of vasopressor agents in the treatment of various forms of shock has been criticized on the grounds that these substances increase vasoconstriction to an undesirable degree and potentiate shock by further stagnation of blood(16). To the contrary, this study in dogs has shown that a pressor drug augments rather than decreases venous return under the circumstances studied.

Conclusions and summary. The early shock produced in the dog by the injection of Gram-negative endotoxin is the result of venous pooling and greatly diminished venous return. Intravenous administration of metaraminol (Aramine) prevented further pooling. Venous return was effectively increased and the mechanism through which the shock was initiated was therefore counteracted.

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Effect of Dietary Fat and Fatty Acid on Fecal Excretion of a Calcium Oleate Phosphate Complex.* (22560)

Leon Swell, E. C. Trout, Jr., Henry Field, Jr. and C. R. Treadwell.
Veterans Administration Center, Martinsburg, W. Va., and Department of Biochemistry, School of Medicine, The George Washington University, Washington, D.C.

Kim and Ivy(1) and Pihl(2) reported that rats fed an oleic acid diet excreted large amounts of phospholipid in their feces. Palmitic acid and corn oil were found to have a mild stimulatory action on phospholipid excretion(1). These authors did not analyze the lipid but assumed that it was phospholipid since it contained phosphorus and could be precipitated from ether solutions with acetone. In the course of work on sterol absorption in these laboratories(3) this material was also encountered in fecal extracts when rats were fed oleic acid diets, and it became of interest to characterize this lipid(4). Chemical and infra-red analysis revealed that the "phospholipid" was a salt containing 2 atoms Ca^{++}, 1 mole $HPO_4^{=}$, and 2 moles oleic acid. It had a pK of 6.1 and was readily dissociated by acid, but was not cleaved by base. A lipid of this composition has not been previously reported and it is of particular interest in that it contains phosphorus and the solubility properties of some phospholipids. P^{32} experiments indicated that the lipid was formed predominantly in the gut from dietary calcium, phosphorus, and oleic acid. The following study was conducted to explore further the formation of this complex in the intestine and also to determine what effect its formation would exert on fat absorption.

Methods and materials. Rats weighing 175-200 g were housed in individual cages arranged for quantitative collection of feces. The diet in slight excess of the daily requirement was weighed and placed in food cups. The diet remaining the following day was weighed and the food intake calculated by difference. The diet consisted of the following: 20% casein, 24% corn starch, 24% glucose, 25% fat or fatty acid, 2% methyl cellulose, 5% Hubbell-Mendel-Wakeman salt mixture, and adequate amounts of crystalline vitamins. An additional 25% carbohydrate was added to the control diet without fat. The rats were divided into 6 groups and each received a different fat or fatty acid in their diet as indicated in Table I.

The diets were fed for 8 days, the feces collected daily, and pooled in each group. The feces of each group were ground and extracted 4 times with boiling 2:1 chloroform-methanol. The extract was washed with water, dried over Na_2SO_4, and filtered. An aliquot of this original extract was taken for analysis and then the remainder of the extract was concentrated to 200 ml in an atmosphere of nitrogen. The phosphorus-containing lipid was precipitated from the chloroform extract once with acetone and then 4 times with methanol. Total lipids were determined on the original extract and on subsequent precipitations by drying at low temperature to constant weight. The amount of the phosphorus-containing lipid present in feces is based on the analysis and weight of the lipid after 5 precipitations. Phosphorus, calcium, and fatty acid content of the lipid were determined on the original extract and at each precipitation as previously described (4). Apparent fat absorption was determined from the dietary fat intake and excretion values, taking the control excretion of the fat-free group into account.

Results. The results are shown in Table I. The control group (F-1) excreted only a trace of lipid-P in their feces. A comparison of the dietary fats shows that corn oil and olive oil stimulated the formation of the complex salt, whereas hydrogenated soybean oil had virtually no effect on lipid-P excretion. Corn oil and olive oil were well absorbed, 95.7% and 97.1% respectively, and hydrogenated soybean oil poorly absorbed, 56.9%. In the case of corn oil and olive oil, the phosphorus-

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TABLE I. Effect of Dietary Fat and Fatty Acid on Fecal Excretion of a Calcium Oleate Phosphate Complex.

Group	No. rats	Dietary fat (25%)	Fat intake per day per rat, mg	Fecal lipid excretion/day/rat — Total lipid, mg	Lipid P,* mg	COP,† mg	Non-COP lipid, mg	Apparent fat absorption,‡ %
F-1	7	None	0	26	Trace	Trace	26	
F-2	7	Hydrogenated soybean oil	2433	1074	"	"	1074	56.9
F-3	7	Palmitic acid	2433	450	.57	7	443	82.6
F-4	6	Corn oil	2490	133	1.00	17	116	95.7
F-5	7	Olive "	2477	98	.90	10	88	97.1
F-6	7	Oleic acid	2491	774	12.52	658	116	70.0

* Determined on original chloroform-methanol extract.
† Calcium oleate phosphate complex. Determined by weighing after 5 pptns. by methanol.
‡ Taking control excretion (Group F-1) into account.

containing lipid accounted for approximately 10% of the total lipid excretion. Of the dietary fatty acids, palmitic and oleic, oleic greatly increased lipid-P excretion. This excretion was above palmitic or any of the fats tested. Palmitic acid had only a slight stimulatory action (.57 mg/day), whereas oleic increased lipid-P excretion to 12.52 mg/day or 658 mg/day of the lipid. This increase accounted for 85% of the total lipid excreted and its formation greatly impaired the absorption of oleic acid (70.0%).

Discussion. The present study has confirmed and extended the observations of Kim and Ivy(1). A comparison of the effect of the different fats on the formation of the calcium fatty acid phosphate complex reveals that the unsaturated fats, corn oil, and olive oil, were more effective in formation of this lipid than the saturated fat, hydrogenated soybean oil. Also, of the fatty acids tested, oleic was far more effective in stimulating formation of the complex than palmitic acid. In the previous study(4) in which the lipid was characterized, it was found that essentially all of the fatty acid was oleic. Thus, this acid would appear to be relatively specific for formation of this complex. Corn oil and olive oil would contribute a small amount of oleic acid during their hydrolysis in the intestine for formation of this lipid complex, whereas the hydrogenated fat would contribute very little oleic acid.

It is apparent that oleic acid absorption is definitely impaired due to formation of this lipid; there being only 70% absorption in the present experiment. The absorption of this acid was even less than palmitic acid (82.6%) which is unusual since unsaturated fatty acids have been shown to be better absorbed than the saturated ones. However, the conditions utilized for determining oleic acid absorption have generally been based on single feedings of the fatty acid alone. It would appear that the feeding of the fatty acid in a diet would more nearly approach physiological conditions of absorption. It is also apparent that dietary phosphorus and calcium absorption are impaired when an oleic acid diet is fed. Based on the dietary intake and excretion of calcium and phosphorus, it was calculated that 50% of the calcium and 50% of the phosphorus were lost in the feces as part of the calcium fatty acid phosphate complex.

At present, it is not known how this lipid is formed in the intestine and what, if any, role it has in fat absorption. Since it is formed predominantly with free unsaturated fatty acid in the intestine, it would appear that very little free unsaturated fatty acid is formed during the digestion of corn oil and olive oil.

Summary. The excretion of a calcium oleate-phosphate lipid complex in the feces of rats was studied under various dietary conditions. Diets containing 25% of either olive oil, corn oil, hydrogenated soybean oil, palmitic acid or oleic acid were compared with a fat-free basal diet. Oleic acid was approximately 70 times more effective in stimulating formation of lipid than either corn oil or

olive oil. Palmitic acid had a very slight effect, whereas hydrogenated soybean oil had no effect. The absorption of oleic acid was much lower than that of palmitic acid, corn oil or olive oil, perhaps due to the formation of the lipid. The significance of the fecal lipid is discussed.

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Life Span of the Duck and Chicken Erythrocyte as Determined with C^{14}. (22561)

KIRKLAND C. BRACE AND PAUL D. ALTLAND.

National Cancer Institute and National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Department of Health, Education and Welfare.

Carbon 14 labeled glycine has been widely used to determine the life span of the nonnucleated erythrocytes of man(1), and several of the lower mammals(2-4) but apparently has not been used to study the life span of the nucleated erythrocytes of the bird. Shemin(5) using N^{15} and Hevesy *et al.*(6) using P^{32} found the life span of the chicken erythrocyte to be 28 days. McConnell *et al.* (7) using Se^{75} to label the cells, determined the life span of the duck erythrocyte to be 11.7 days. This present study was undertaken to provide additional information on the erythrocyte life span of these 2 species by the use of C^{14} and to evaluate the applicability of this material in the measurement of the life span of nucleated erythrocytes.

Methods. Glycine-2-C^{14} was injected intravenously into the wing vein of the birds. Two adult female chickens were given 17 μc per kilo of body weight and 2 adult male ducks were given 35 μc per kilo. Two additional ducks were given the same dose intraperitoneally for studies of the rate of uptake. One half ml of blood was obtained at regular intervals after the initial injection. Samples containing 0.1 ml of packed cells were prepared by washing the cells 3 times with saline and precipitating the protein with 10% trichloroacetic acid. The precipitate was washed and plated onto a 1 inch copper planchet and counted in a gas flow proportional counter.

Results. The radioactivity of the red cell protein of the duck and chicken is shown as a function of time in Fig. 1. There was an unusually rapid uptake of the labeled carbon into the protein of the duck cells. This response was independent of the method of injection because the rapid uptake was also observed following intraperitoneal injection of the glycine. The character of the uptake curve and the double hump noted at the peak of the curve suggested that approximately 10% of the glycine α-carbon incorporation was in the red cells already in the peripheral circulation. The sigmoidal decay was typical of that observed in man and the mammals where only a relatively homogenous age group of cells forming in the marrow are labeled. We therefore assumed that the lower plateau and decay curve represent the labeling of the more or less homogenous age group forming in the marrow at the time of injection. An analysis of the curves gave a value of 44 days from the initial labeling until 50% of the cells were destroyed with half of the labeled cells destroyed in the interval $\pm$ 5 days. The mean survival time, which represents the average time during which a given cell may exist in the peripheral circulation, is ordinarily computed as the interval between the appearance and disappearance of 50% of the labeling. If we ignored the unusually rapid uptake the value was 42 days. If we assumed that the

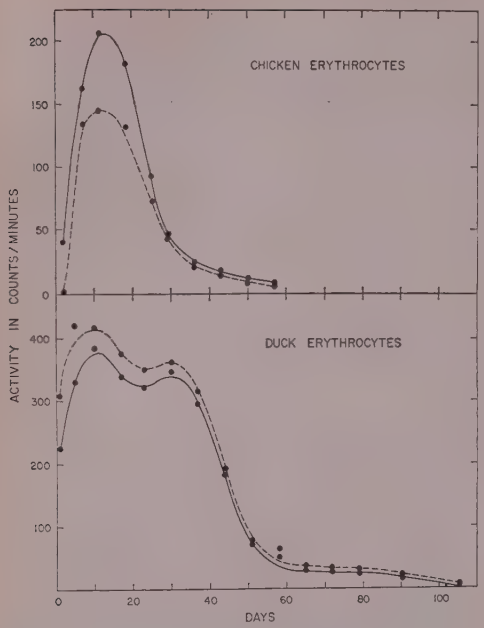

FIG. 1. Change in radioactivity of red cell protein after intravenous inj. of glycine-2-C^{14} in duck and chicken. Solid and broken lines represent individual animals.

uptake of C^{14} by the cells in the duck marrow was similar to that found in the chicken, the mean survival time of the red cells of the duck was reduced to about 39 days.

The slow uptake of the glycine α-carbon into the protein of the chicken erythrocyte suggested that the labeling of the cells other than those forming in the marrow contributed relatively little to the total activity. The absence of a well defined plateau was due principally to an apparent shorter life span. Fifty per cent of the cells were destroyed 25 days after injection with half of the cells destroyed in the interval $\pm$ 4 days. The mean survival was reduced to 20 days by the prolonged period of uptake.

Discussion. The longevity of the erythrocytes of the chicken and duck obtained by the use of C^{14} differs from the values obtained by other methods. The lower value of 20 days for the chicken as determined with C^{14} contrasts with the 28 days obtained with N^{15} and P^{32}(5,6). The higher value may be due to the fact that the uptake of the labeling material in their study extended over a period of several days and depended upon the absence of or a correction for the metabolism of cells in the circulating blood. Our data suggest that the glycine α-carbon metabolism of the circulating cells was insignificant in the chicken and therefore our values represent a more direct determination.

The value of 39 days for the longevity of the duck erythrocyte obtained with C^{14} is in marked contrast to that of 11.7 days obtained with Se^{75} by McConnell *et al.*(1). These investigators transfused cells labeled with a toxic material, a technic which provides only a minimum value for the life span. The mean life span was probably nearer the value obtained with C^{14}. The very rapid uptake of C^{14} during the first 2 days after injection in our study suggested that possibly some of the isotope was incorporated into the protein of the cells already in the peripheral circulation. *In vitro* studies(8) have shown that glycine is utilized in the synthesis of hemoglobin in the circulating duck cells. Extensive studies on the turnover of the α-carbon glycine in the mammal indicate, however, that reutilization of the labeled carbon from hemoglobin is very small, and the long decay curve can be ascribed to the utilization of labeled glycine from the body pool for the formation of new cells(9). It is of interest to contrast the relatively short life span of the nucleated bird erythrocytes with the long life span of more than 11 months obtained for the nucleated erythrocytes of the turtle(10) by the use of the same method. The definitive life span of the turtle erythrocyte and other cold blooded animals remains to be delineated.

Summary. Measurements of the life span of the erythrocyte with glycine-2-C^{14} give a value of 20 days for mean survival time for the chicken and 39 days for the duck. The uptake of the α-carbon of glycine into the cell proteins in the chicken was similar to that observed in the mammals. The shape of the decay curve was typical of that observed when a homogeneous group of red cells are labeled in the marrow, and gave little evidence of reutilization of the labeled carbon. The utilization of glycine α-carbon by the duck cells was

similar to that observed in the chicken except for the rapid uptake which gave some evidence of uptake by the cells in the peripheral circulation.

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Effect of Chlorpromazine and Dibenzyline on Bacterial Toxins. (22562)

HOWARD E. NOYES, JAY P. SANFORD* AND RUSSELL M. NELSON.†
(With technical assistance of Edward B. Phillips.) (Introduced by R. W. Clarke.)
Department of Experimental Surgery, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D. C.

Various bacterial toxins, particularly the endotoxins from "coliform" bacteria and the exotoxin of *Clostridium perfringens,* have been considered important factors in the pathogenesis of the decompensated phase of prolonged hypotension (irreversible shock) (1-3). Premedication with a number of pharmacologic agents particularly chlorpromazine and dibenzyline, has been shown to decrease the mortality in the shock syndrome, whereas the same treatment initiated after the onset of shock has been ineffective(4-6). The mechanism of protection has not been elucidated. Chlorpromazine recently has also been reported to offer protection against "endotoxemia" in man and experimental animals and to be useful in the management of tetanus (7-10). The effect of either chlorpromazine or dibenzyline in attenuating lethal shock might be the result of direct action on bacterial toxins or alteration of the host response to them. A study of the effects of chlorpromazine and dibenzyline in animals injected with a variety of bacterial toxins, including those which may play a role in the pathogenesis of irreversible shock, was undertaken and is the basis of this report.

Methods and materials. White Swiss mice, Bagg Strain, each weighing 16-18 g were used. Food and water were readily available to the animals throughout the experiments. The characteristics of the toxins used are listed in Table I. Dosages of the various toxins to range from an LD_{40} to an LD_{70} were estimated by preliminary titrations. All toxins were diluted in 0.9% NaCl solution and, if not sterile, sterilized by filtration through sintered glass. The intravenous route of administration was used with the toxin from *S. dysenteriae,* the subcutaneous route with the tetanus toxin, and the intraperitoneal route with the other toxins. Chlorpromazine and dibenzyline‡ were obtained as sterile solutions. The therapeutic regimens generally corresponded to those reported to be effective in the shock syndrome. The dose of chlorpromazine was 33.5 μg per injection (2 mg/kg) while that of dibenzyline was

* Present address: Duke University School of Medicine, N. C.

† Present address: University of Utah College of Medicine, Salt Lake City.

‡ Both obtained through the courtesy of the Smith, Kline, and French Laboratories.

TABLE I. Sources of Toxin Used in This Study.

Organism	Type of toxin	Obtained from	Chemical composition	Extraction procedure
Cl. botulinum	Neurotoxin	Wright(11)	Protein	Acid precipitation of culture filtrate
Cl. perfringens	Culture filtrate	Lederle Lab.	?	Culture filtrate
Cl. tetani	Neurotoxin	Anonymous	Protein	Ammonium sulfate precipitation of autolysed culture
A. aerogenes	Endotoxin	Noyes(12)	Lipopolysaccharide	Trichloracetic acid extraction followed by ethanol fractionation
E. coli	"	" (12)	"	
Past. pestis	Murine toxin	Ajl(13)	Protein	Ammonium sulfate fractionation of autolysed culture followed by electrophoretic separation
S. typhosa	Endotoxin	Webster(14)	Lipopolysaccharide	Trichloracetic acid extraction
S. dysenteriae	Neurotoxin	Branham(15)	Protein	Autolysed cells

17 μg (1 mg/kg). These dosages were contained in 0.5 ml of sterile 0.9% saline solution. Both were administered intraperitoneally. The mice were premedicated 24 hours and 2 hours before administration of toxin in most experiments. Treatment was repeated at 24-hour intervals in most studies until termination of the experiment. All experiments were continued until no additional deaths occurred. Control mice were treated with saline solution according to the same schedule. *In vitro* experiments were carried out to determine if either chlorpromazine or dibenzyline exerted direct "antitoxic" effects. Dibenzyline was mixed with the *S. dysenteriae* neurotoxin to give a dibenzyline concentration of 100 μg/ml or 33 μg/ml with approximately an LD_{70} of toxin/ml. Control mixtures were diluted with saline solution. The mixtures were incubated at 37°C for 6 hours. Aliquants were removed immediately after mixing and during incubation for assay of toxicity in mice.

The therapeutic effectiveness of dibenzyline against *S. dysenteriae* toxin was studied by treating groups of mice either 3 or 6 hours after administration of toxin. The effectiveness of chlorpromazine against *E. coli* endotoxin was similarly studied at 2, 4, and 6 hours after injection of toxin.

Statistical evaluation of the differences in mortality between experimental and control animals, using the method of Chi square, was made. Most of the experiments were repeated at least twice. These individual experiments were combined for statistical evaluation.

Results. Dibenzyline and chlorpromazine were each effective in decreasing the mortality from various toxins. However, the 2 agents were not effective against the same toxins (Table II). Premedication and treatment with dibenzyline afforded protection to mice injected with toxins of *S. dysenteriae, P. pestis,* and *Cl. tetani.* The effectiveness of dibenzyline on endotoxin derived from various species of the enterobacteriaceae was variable. It afforded significant protection against endotoxin derived from a strain of *A. aerogenes,* offered no protection against endotoxin derived from a strain of *E. coli,* and increased the mortality from endotoxin prepared in a similar fashion from *S. typhosa.* Dibenzyline offered no protection to animals injected with toxins derived from *Cl. botulinum* or *Cl. perfringens.* Chlorpromazine afforded protection against the endotoxins of *E. coli* and *S. typhosa,* but was not protective in a single experiment using endotoxin derived from *A. aerogenes.* Chlorpromazine was very effective in 2 groups of mice injected with endotoxin isolated from a strain of *S. typhosa* ($P = 0.001$). However, in a third experiment using the same *S. typhosa* toxin, an increased mortality occurred in the chlorpromazine-treated group of animals (Table II). Chlorpromazine had no protective effect against the other toxins studied. The administration of chlorpromazine actually increased the mortality rate in mice injected with *Cl. perfringens* toxin (Table II). The possibility that the protective effects of these drugs were the result of direct interaction of toxin and drug was studied *in vitro.* The lethality of the Shiga neurotoxin was diminished after 6 hours

of incubation with dibenzyline (Table III). It was also noted that a precipitate formed when dibenzyline was mixed with the *S. dysenteriae* neurotoxin. The precipitate was resuspended immediately prior to injection.

Administration of chlorpromazine and *E. coli* endotoxin to mice immediately following mixing or after 6 hours of incubation decreased mortality rate (Table III). A direct antitoxic action of chlorpromazine could not be assessed because it was effective even when administered after the endotoxin. The effectiveness of these drugs administered at intervals after injection was compared to their effectiveness following administration prior to toxin. Chlorpromazine administered simultaneously with, or 2 hours after, the injection of an LD_{80} of *E. coli* endotoxin afforded marked protection (0 hours—$P<0.001$, 2 hours—$P<0.01$) (Fig. 1). There was a suggestion of protection at 4 hours ($P<0.20$). Dibenzyline administered either 3 or 6 hours after *S. dysenteriae* toxin offered no protection.

Discussion. Pretreatment of mice with chlorpromazine or dibenzyline conferred protection against a variety of bacterial toxins. Protection might have been the result of alteration in the vascular response of the host to the bacterial toxins. Both chlorpromazine

TABLE II. Mortality* of Mice from Bacterial Toxins following Premedication with Chlorpromazine or Dibenzyline.

	Chlorpromazine					Dibenzyline				
Source of toxin	Treated	%	Controls	%	P‡	Treated	%	Controls	%	P
S. dysenteriae	64/ 89*	72	60/ 83	72		50/100	50	68/100	68	<.01
	47/100	47	44/100	44						
A. aerogenes	18/101	18	25/ 90	28		9/ 98	9	20/100	20	
						26/107	24	40/100	40	<.001
						27/ 50	54	36/ 50	72	
						40/ 60	68	48/ 80	60	
						46/ 50	92	49/ 50	98	
E. coli	78/100	78	97/104	93	<.001	67/100	67	75/100	75	
	67/100	67	96/107	90		74/100	74	78/100	78	
						10/ 50	20	18/ 50	36	
S. typhosa	40/100	40	72/100	72		69/104	66	50/105	48	
	60/100	60	48/100	48	<.01	61/ 97	63	53/100	53	<.01
	87/102	85	99/101	98		23/ 50	46	21/ 50	42	
P. pestis	26/100	26	19/100	19		88/100	88	96/100	96	<.05
	77/ 93	83	82/ 93	88		87/100	87	92/100	92	
Cl. botulinum	46/100	46	36/100	36		90/102	88	92/102	90	
Cl. perfringens	46/100	46	33/100	33		67/102	66	66/102	65	
	48/100	48	40/100	40	<.05†	65/100	65	54/100	54	
Cl. tetani	71/100	71	44/100	44		93/100	93	99/100	99	<.01
	82/100	82	98/100	98		69/ 99	70	82/ 97	85	

* Dead/injected.
† Mortality increased.
‡ Probability values computed from all data for each toxin. Values less than >.05 have been omitted.

TABLE III. Mortality from Bacterial Toxins following Incubation *In Vitro* with Chlorpromazine or Dibenzyline.

			Mortality* after incubation *in vitro*			
Source of toxin	Agent	(μg/mouse)	0 hr	P	6 hr	P
S. dysenteriae	Dibenzyline	100	36/40		23/40	
	Saline	—	38/40	>.50	37/40	<.001
E. coli	Chlorpromazine	100	3/23		0/23	
		33	17/58		9/58	
	Saline	—	39/58	<.001	31/58	<.001

* Dead/injected.

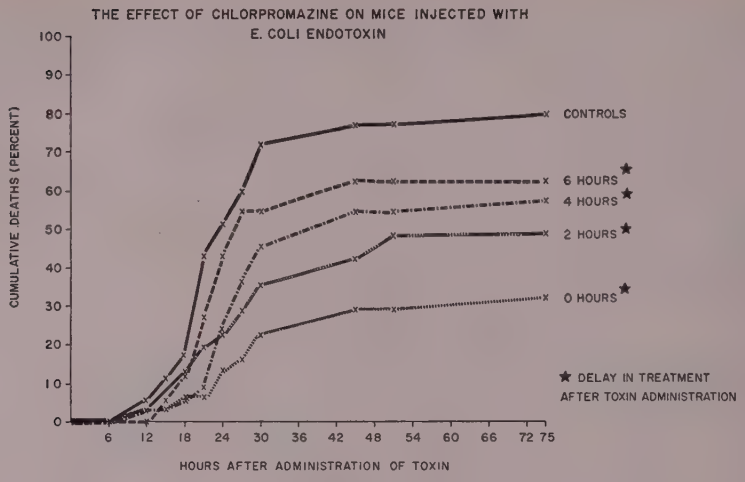

FIG. 1.

and dibenzyline have potent adrenolytic actions. Chlorpromazine may also have a central nervous system action, in that blood pressure can be lowered by amounts which do not block the vasopressor effects of epinephrine. The injection of minute doses intracisternally into rhesus monkeys caused a fall of blood pressure and blockade of the carotid pressor reflex, whereas the pressor response to the systemic injection of adrenaline remained unaffected(16). However, a direct action on the toxin cannot be eliminated, since inactivation of one bacterial toxin occurred upon incubation *in vitro*.

The protective effect of these agents in hemorrhagic or traumatic shock may be the result of alterations in the response to bacterial toxins. Both chlorpromazine and dibenzyline were effective against various endotoxins, but their effectiveness was not uniform. Dibenzyline failed to protect against endotoxin prepared from a strain of *E. coli* or *S. typhosa* when it did afford protection against endotoxin prepared in a similar fashion from a strain of *A. aerogenes*. Neither agent afforded any protection against the toxin of *Cl. perfringens*. A common antitoxic action seems unlikely as the mechanism of protection by chlorpromazine and dibenzyline in hemorrhagic shock, because of the discrepancies noted in their effectiveness against those toxins considered as possible factors in the pathogenesis of irreversible shock. Separate mechanisms of action by chlorpromazine and dibenzyline also were suggested by a series of experiments on their protective effect in hemorrhagic shock following partial or complete abdominal evisceration(17). Protection by one agent and not the other should not have been observed if their modes or sites of action were similar.

The effectiveness of chlorpromazine and dibenzyline in various specific bacterial toxemias should be stressed, in addition to any interrelationships between chlorpromazine, dibenzyline, bacterial toxins, and shock. Dibenzyline decreased mortality in animals injected with Shiga neurotoxin, plague toxin, and tetanus toxin. Chlorpromazine was primarily effective against the endotoxins of Gram-negative bacteria, including *S. typhosa*. Chlorpromazine not only was effective when administered before injection of endotoxin, but was also very effective when treatment was delayed until several hours after injection of endotoxin. The mechanism of the protection against endotoxin derived from the enterobacteriaceae is not known. However, epinephrine has been demonstrated to increase susceptibility of the host to endotoxin(18). The adrenolytic action of chlorpromazine may account for some of the protective effect, though dibenzyline, which has a similar action, would also be expected to be effective

and was not.

Summary and conclusions. 1. Chlorpromazine and dibenzyline were tested in mice for their ability to afford protection against a variety of bacterial toxins. 2. Premedication and treatment with chlorpromazine significantly decreased the mortality from endotoxins derived from *Escherichia coli* and *Salmonella typhosa*. Similar treatment with dibenzyline decreased the mortality from toxins derived from *Shigella dysenteriae, Aerobacter aerogenes, Pasteurella pestis* and *Clostridium tetani*. 3. Chlorpromazine afforded significant protection when administered 2 hours following the injection of endotoxin derived from *Escherichia coli*. Premedication was not necessary. 4. Premedication with chlorpromazine increased the mortality from the toxin of *Clostridium perfringens*, likewise premedication with dibenzyline increased the mortality from the endotoxin derived from *Salmonella typhosa*.

The authors wish to express their appreciation to the workers who so kindly made available the various toxins used in this study.

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Concentration of ACTH in Cavernous Sinus and Peripheral Arterial Blood in the Dog.* (22563)

WILLIAM F. GANONG† AND DAVID M. HUME.‡
(Introduced by G. W. Thorn.)
Laboratory for Surgical Research, Harvard Medical School and the Surgical Service, Peter Bent Brigham Hospital, Boston.

Collection of effluent blood from the pituitary has been impractical because of its position in the skull and the early divergence of cerebral venous drainage into many channels. Since blood drains directly from the pituitary into the cavernous sinus, at least in some species, samples of blood from this location, although contaminated by blood from the ophthalmic veins, would be expected to contain a high concentration of pituitary hormones (1). The present paper describes a simple and reliable technic for obtaining blood from the

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† Formerly U. S. Public Health Service Research Fellow for the National Institute of Neurological Diseases and Blindness. Present address: Department of Physiology, University of California, Berkeley.

‡ Scholar of the American Cancer Society.

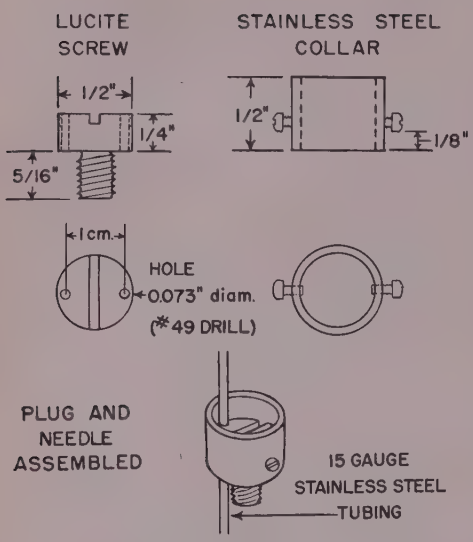

FIG. 1. Plug and guide needle for cavernous sinus puncture.

cavernous sinus in the dog, and presents evidence that the concentration of ACTH in this blood is much higher than that found in peripheral arterial blood.

Materials and methods. Adult mongrel dogs weighing 10-14 kg were used in this study. They were maintained on a diet of Ken-L-Biskit.§ Penicillin, 300,000 units daily, was given intramuscularly for one week after the animals were operated upon. The plastic button with a screw stem and stainless steel collar used to hold the guide needle in place is illustrated in Fig. 1. The stereotaxic machine used in these studies is described in detail elsewhere(2). Implantation of the plastic button was performed under aseptic conditions using intravenous pentobarbital anesthesia. The dog's head was placed in the stereotaxic instrument, and a curved incision was made through the scalp. The incision started in the mid line between the eyes and as it extended posteriorly it curved to one side, passing 3 cm lateral to the mid line opposite the center of the head, and returning to the mid line posteriorly, ending about 3 cm anterior to the occipital protuberance.

A lateral skull x-ray was taken and a reference point was located at the junction of the sloping inner table of the sphenoid bone and the floor of the optic foramen. The carrier of the instrument was centered above a point 1.3 cm posterior to the reference point. The previously outlined skin flap was elevated and retracted. Using the instrument, a hole 7/32 of an inch in diameter was drilled in the mid line down to the dura. The hole was tapped with a square ended ¼ inch #20 thread tap, and the button was inserted and tightened, so that a line drawn through the two holes in the button was perpendicular to the sagittal suture. Two short pieces of stainless steel wire were passed under the head of the screw, one forming a loop toward the front and the other toward the back of the head. The skin flap was then replaced after a round hole was cut in it to accommodate the head of the screw, and the ends of the stainless steel wire were brought thru the skin close to the hole and tied to each other, thus securing the skin in place about the plastic button. The carrier was next moved 5 mm to one side, a #49 drill was inserted, and using the holes in the plastic button as a guide, a hole was drilled thru the skull. A 3 inch length of 15 gauge stainless steel tubing‖ with a square-cut smoothed end was inserted thru the hole and thru the brain to, but not thru, the superior wall of the cavernous sinus. This structure was identified by feeling an elastic resistance to the tip of the needle. The stainless steel tubing was cut off *in situ,* leaving ⅛ of an inch of tubing projecting above the button. The stainless steel collar was next slipped over the plastic button and the set screws tightened to hold the needle in place. A bent piece of 18 gauge hypodermic needle was inserted in the top of the 15 gauge needle as a trochar, the wound was sutured, and the animal was allowed to recover for at least one week. Guide needles were put down to only one of the 2 cavernous sinuses at a time. If, at a later date, it was desired to tap the other cavernous sinus, a hole was drilled on the outer side and a needle was inserted in the same manner as on the first side. When the

§ Quaker Oats Co., Chicago, Ill.

‖ Obtained from MacGregor Instrument Co., Needham, Mass.

animals had fully recovered, cavernous sinus blood was obtained by removing the trochar, and inserting a sterile 18 gauge lumbar puncture needle down the guide needle and into the cavernous sinus. Blood was collected into heparinized syringes, care being taken to exert very little pressure on the syringe so as not to reverse the flow of blood in the sinus. In a few instances, the dog was heparinized and laid on his back. Cavernous blood was then drained by gravity thru the lumbar puncture needle into a test tube. The buttons were well tolerated, and the small amount of reaction around the device subsided in about a week.

In the present studies, 25 cc of cavernous sinus blood and 50 cc of femoral arterial blood were drawn simultaneously from normal and adrenalectomized dogs under ether anesthesia. The animals were subjected to various degrees and durations of surgical stress. Samples were also obtained from the cavernous sinus of unanesthetized unrestrained dogs. Puncture of the sinus is occasionally somewhat painful, but the pain, when present, lasts only for an instant, and thereafter the animals appeared to show no ill effects from the puncture. Subsequent removal of the puncture needle was not associated with significant hemorrhage in any instance, presumably because the elastic wall of the sinus was not prone to leak and because the pressure within the sinus was so low. As many as 5 samples were taken at 2-3 day intervals from some of the dogs, and aside from the anemia due to blood removed, no untoward effects were noted.

Two dogs in whom cavernous sinus guide needles had been inserted were subjected to cannulation of the right adrenal according to the technic of Hume and Nelson(3), and the animals were permitted to recover. Three days later, adrenal venous blood samples were obtained while the puncture needle was inserted into the cavernous sinus. Adrenal venous samples were taken before, and 5, 10, and 30 minutes after, the insertion of the needle into the cavernous sinus.

One dog in whom a cavernous sinus guide needle had been implanted was subjected to laparotomy under pentothal-induced ether anesthesia. A cavernous sinus blood sample was obtained, and immediately thereafter his pituitary was removed thru the trans-buccal approach. One half hour later, another cavernous sinus blood sample was obtained.

TABLE I. ACTH Content (Milliunits per 100 cc Plasma) of Cavernous Sinus and Femoral Artery Blood Samples Drawn Simultaneously at Arbitrary Times during Various Experimental Conditions.

Dog	ACTH content, milliunits/100 cc plasma	
	Cavernous sinus blood*	Femoral artery blood*
311	18	4.0
X	50	2.0
18	12	0 †
115	40	10
312	18	2.0
148	20	0 †

* ACTH content determined by inj. the plasma from 25 cc of cavernous sinus blood and 50 cc of femoral artery blood.

† ACTH could not be detected in the volume of plasma tested.

All cavernous and arterial samples were placed in heparinized tubes, centrifuged immediately, and the plasma stored in the frozen state until used. Determination of the ACTH content of these samples was performed by the technic of Nelson and Hume(4). The 17-hydroxycorticosteroid content of the adrenal venous blood was determined by the method of Nelson and Samuels(5).

Results. The ACTH content of the simultaneously obtained cavernous sinus and arterial blood samples is listed in Table I. It is apparent that the concentration of ACTH in the cavernous sinus blood was much greater than it was in the femoral artery blood. The ACTH concentration in the cavernous sinus blood of the animal subjected to laparotomy and hypophysectomy was 28 milliunits per 100 cc of plasma before hypophysectomy, and was not detectable one-half hour after. It would appear, therefore, that the substance being assayed was pituitary in origin. Repeated cavernous sinus samples during other surgical procedures without removal of the pituitary show consistent high levels for at least 60 minutes. The effect of cavernous sinus puncture on adrenocorticosteroid output is illustrated in Table II. Insertion of the

TABLE II. Adrenal Venous Blood 17-Hydroxycorticoid Output (μg/Min.) in Response to Puncture of the Cavernous Sinus.

	Dog No. 312	Dog No. 286
Control	3.3	.2
5 min. after puncture	15.6	12.4
10 " " "	1.2	9.9
30 " " "	2.3	.2

needle in these 2 dogs was associated with a definite but small and transient rise in corticoid output.

Discussion. Hume and Nelson(3) have recently published a technic for obtaining adrenal venous effluent blood in the unanesthetized animal. This method for studying adrenal hormone output has two advantages over the present technic for studying pituitary hormone secretion. In the first place, the adrenal has a single effluent vein, making it possible to obtain all of the undiluted adrenal venous blood. In the dog the cavernous sinus at the level at which it is tapped drains not only the pituitary but also the ophthalmic veins. Secondly, the present method of obtaining cavernous sinus blood does not permit measuring the rate of pituitary blood flow. Therefore, actual measurements of ACTH output per unit time are as yet beyond the reach of this method. In some species, much of the pituitary venous blood passes through the sphenoid bone via the emissary veins. Detailed descriptions of the venous outflow from the pituitary in the dog are not available. However, the present data showing a much greater concentration of ACTH in cavernous sinus than in peripheral blood indicate that in the dog at least a part of the pituitary drainage is into the cavernous sinus. Furthermore, the emissary vein in this species is extremely small and often altogether absent. The advantage of the present technic lies in the high concentration of ACTH, and presumably of other pituitary hormones, in the cavernous sinus blood. This high concentration permits the use of smaller volumes of blood for hormone measurement, and may make possible the detection of ACTH under circumstances where its detection in the peripheral blood is difficult or impossible. Furthermore, although insertion of the cavernous sinus needle has been demonstrated to be a mild stress, it is a short lasting one. If the needle is inserted and left in place for 20-30 minutes, the effects of its insertion will have worn off so that cavernous sinus blood can be obtained in the unanesthetized, unrestrained normal animal. Studies of the effect of trauma and various other conditions on cavernous sinus ACTH content are under way in this laboratory at present.

Summary. A simple and reliable method of obtaining blood from the cavernous sinus of the dog has been presented. The level of ACTH in this blood under a variety of conditions has been found to be many times higher than the concentration in peripheral arterial blood. Hypophysectomy causes a fall to zero in the level of ACTH in this blood. With this technic, insertion of the needle into the cavernous sinus is shown to be a definite but transient stress.

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Recovery from Stress in Thyroidectomized Rats.* (22564)

Toh-Bee Syn and Melvin Hess.†
Department of Anatomy, Emory University, Ga.

It has been reported by Hess *et al.* (1) that the increased adrenal response of thyroidectomized rats to stress is not due to a difference in pituitary ACTH release. The following experiments were designed to determine whether the lower adrenal ascorbic acid values after stress in operated animals are due to a failure of the hypothyroid rat to metabolize the vitamin. Is the adrenal ascorbic acid in stressed-thyroidectomized rats lower than controls because they cannot rebuild their adrenal content? If such is the case, then one would expect to find lower adrenal ascorbic acid values in operated animals at various periods of time of recovery from a stress.

Methods. Male rats of the Holtzman strain were thyroid-parathyroidectomized at an age of 6 weeks, and together with intact animals, maintained on Purina Laboratory Chow pellets and tap water *ad libitum* for an additional 6 weeks. Some of the animals from each group then were injected intravenously with ACTH (Armour Corticotropin) at dosages of 5 milliunits and 15 milliunits/100 g of body weight, under Nembutal anesthesia. Saline-injected animals (0.1 cc/100 g of body weight) from both groups served as controls. Groups of operated and intact rats were sacrificed at 1, 6, 12 and 24 hours after treatment, left adrenal and thymus glands weighed on a torsion balance, and adrenal glands assayed for ascorbic acid content according to the method of Roe and Kuether(2).

Results. One hour after injection of both doses of ACTH, significant depletions of adrenal ascorbic acid are observed in both operated and intact rats compared to saline-injected controls (Table I). Only in the adrenal of the thyroidectomized rat, at the higher dose level, is the depletion of ascorbic acid more striking. The depletion is significantly lower than in similarly treated intact rats.

Adrenal ascorbic acid analyses at periods of 6, 12 and 24 hours after treatment (Table I) reveal no significant differences between ACTH-injected thyroidectomized and intact animals nor between the ACTH-treated rats and their saline-injected controls (with the exception of one group of intact rats).

In the group of animals sacrificed one hour after treatment, relative weights of adrenal and thymus glands show no significant differences. Six hours after treatment, the intact rats receiving the higher dose of ACTH reveal larger adrenal glands than other intact and operated animals; thymus gland weights do not differ between groups of operated and intact rats. Relative organ weights of animals autopsied 12 hours after treatment show no significant differences in adrenal size between operated and intact rats, but the thyroidectomized animals have significantly smaller thymus glands. The adrenal glands of operated and intact rats 24 hours after treatment show no differences, whereas some decrease in size of the thymus glands of some thyroidectomized rats is evident.

Discussion. That thyroidectomized rats elicit a greater response to a given injection of ACTH, compared to intact animals, is evident from the adrenal ascorbic acid figures reported for operated animals receiving the higher dose of the trophic hormone one hour before autopsy. If these lower values in ACTH-injected thyroidectomized rats are due to a failure of the hypothyroid animal to rebuild the adrenal ascorbic acid after depletion or to destroy the circulating ACTH at a normal rate, then analyses in operated rats at later periods of time should also be lower. Such is not the case. As soon as 6 hours after ad-

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† Present address: Department of Anatomy, University of Pittsburgh, Pittsburgh, Pa.

TABLE I. Adrenal Ascorbic Acid (mg/100 g Tissue) after Graded Doses of ACTH.

Group	Hr after treatment	Saline	ACTH 5 mu dose	ACTH 15 mu dose
Intact	1	333 ± 10†(15)	273‡± 11†(21)	270‡ ± 11† (18)
Thyroidectomized	1	376 ± 21 (23)	239‡± 13 (26)	201‡§± 17 (25)
Intact	6	394 ± 9 (11)	383 ± 10 (13)	386 ± 8 (14)
Thyroidectomized	6	375 ± 9 (14)	406 ± 16 (14)	401 ± 19 (14)
Intact	12	425 ± 7 (16)	397 ± 8 (17)	392‡ ± 7 (17)
Thyroidectomized	12	399 ± 16 (14)	390 ± 15 (14)	417 ± 18 (14)
Intact	24	463 ± 6 (10)	486 ± 7 (12)	465 ± 11 (10)
Thyroidectomized	24	465 ± 17 (11)	498 ± 18 (15)	457 ± 21 (15)

† Stand. error.
‡ Difference from saline-inj. controls statistically significant.
§ " " similarly treated intact rats statistically significant.
Numbers in parentheses are No. of animals in each group.

ministration of ACTH, thyroidectomized rats show the same degree of recovery of adrenal ascorbic acid as intact animals. Likewise, no differences exist at 12 and 24 hours after treatment either. On a formal basis, one can interpret the data to indicate that 6 hours may be a long enough period for the slower process of recovery in the thyroidectomized rats to "catch-up" with that of intact rats. However, this possibility would seem to be less likely.

During the periods of recovery from ACTH injections, all of the operated and most of the intact rats had adrenal ascorbic acid values which did not differ significantly from saline-injected controls. Only one group of intact rats, 12 hours after treatment with the higher dose of ACTH, revealed adrenal ascorbic acid values significantly different from its saline control group. The adrenal ascorbic acid value of this group did not differ from its control by a large margin; the difference, though small, was just statistically significant. However, in no instance during the recovery periods did the adrenal ascorbic acid values of operated and intact rats differ.

The relative weights of the adrenal glands of thyroidectomized and intact rats reveal no significant differences, with the exception of one group of intact animals. The intact rats receiving 15 milliunits of ACTH and autopsied 6 hours later displayed larger adrenal glands than those of thyroidectomized animals sacrificed at the same time. Since the same situation does not persist in the groups of animals autopsied at later periods of time, one can assume that the higher dose of ACTH was no more effective in producing adrenal enlargement in intact than in operated rats at the dose levels and periods of time employed. No explanation on a physiological basis can be offered for the apparent adrenal enlargement in that one group of intact rats.

For the most part, the relative thymus weights of all groups of thyroidectomized animals are less than those of intact rats. In several groups, the differences are statistically significant. Referring to the thymolytic action of adrenal cortical hormones(3,4), the data suggest more active secretion on the part of the adrenals of operated than of those of intact rats.

The lower ascorbic acid values in stressed-thyroidectomized rats are not due to smaller nor inactive adrenal glands, are not due to a difference in amounts of ACTH released from the pituitary gland, and are not due to any failure on the part of the adrenal of the operated rat to rebuild the vitamin. One only can conclude that there is some inherent difference in the response of the adrenal of the thyroidectomized rat to a given stress, as compared to the intact animal, but the basis for this difference has not been determined.

Summary. 1) To determine whether the lower adrenal ascorbic acid value after stress in the thyroidectomized rat is due to failure of the hypothyroid animal to rebuild the vitamin, rats were thyroidectomized, injected with ACTH, and sacrificed at various periods of time following treatment. The adrenal ascorbic acid level was followed during the recov-

ery periods. 2) One hour after injection, operated rats showed a greater depletion of adrenal vitamin than intact animals. If the lower values in operated-stressed rats are due to failure to rebuild ascorbic acid, then analyses of adrenals of operated animals at later periods of time should also be lower. Such is not the case. Six hours after treatment, thyroidectomized rats show the same degree of recovery of adrenal ascorbic acid as intact animals. The basis for the difference in response of the thyroidectomized rat to a given stress has not been determined.

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Use of Copper-Amino Acid Complexing for Determining Amino Acid Esterase Activity.* (22565)

J. J. HAGAN, F. B. ABLONDI AND B. L. HUTCHINGS.
(With the technical assistance of R. E. Clarke.)

Biochemical Research Section, Research Division, American Cyanamid Co., Pearl River, N. Y.

Spies and Chambers(1) described an adaptation of a method for the quantitative determination of amino acids which is based on the formation of a colored copper-amino acid complex. The method is limited to the determination of N-unsubstituted amino acids which form soluble complexes. Since copper does not form a complex with amino acid esters, the method lends itself to the determination of an amino acid liberated as a result of ester hydrolysis. The present paper describes an application of the copper method to the determination of lysine resulting from the enzymatic hydrolysis of lysine ethyl ester. In our laboratory, this procedure was found to be simple and easily adaptable to routine enzymatic analysis.

Materials. Copper phosphate suspension is prepared by the addition of 0.21M cupric chloride to 1600 ml of 0.18M trisodium phosphate with stirring. After centrifugation for 5 minutes at 2000-3000 rpm, the precipitate is washed by resuspension in 2000 ml of 0.1M borate buffer, pH 7.2. The precipitate is collected, again washed and then suspended in 2000 ml of the buffer. Two hundred and forty grams of reagent grade sodium chloride are added and the suspension is adjusted to pH 7.2 with 5N sodium hydroxide. The preparation is similar to that used by Schroeder *et al.*(2). L-Lysine ethyl ester dihydrochloride (LEE) is prepared as described by Werbin and Palm(3) and L-lysine monohydrochloride was purchased from Nutritional Biochemicals. Both were stored in a desiccator over phosphorous pentoxide. Twice recrystallized trypsin (Worthington) was used as the LEE esterase.

Method. Two ml of copper phosphate suspension are added to 2 ml of the test solution contained in a 15 ml conical centrifuge tube. The tube is shaken intermittently by hand for 5 minutes and excess copper phosphate removed by centrifugation for 2 minutes at 2000 rpm. The blue colored supernatant fluid is decanted into a Klett microtube and read in the Klett Summerson Photoelectric Colorimeter using a #66 (Red) filter.

Lysine standard curve. Since many of our hydrolysis experiments were carried out between pH 6.2 and 7.3, we investigated the effect of pH on the color intensity of the copper-lysine complex. Lysine standard curves

* The authors are indebted to Mr. John Poletto for the preparation of LEE hydrochloride and to Mr. C. P. Dunnet for the statistical analysis. We wish to thank Dr. E. C. De Renzo for his many helpful suggestions in preparing this manuscript.

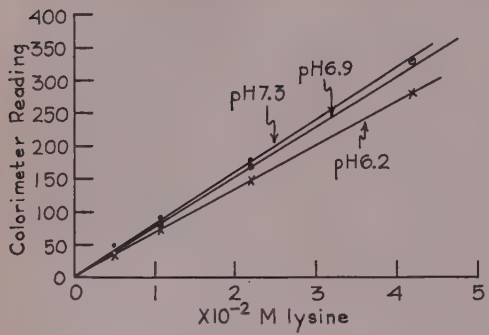

FIG. 1. Effect of pH on the lysine standard curve.

were prepared at pH 6.2, 6.9 and 7.3 in 0.15M phosphate buffers. As shown in Fig. 1, the curves are linear although the color intensity varies with the pH of the copper lysine complex.

An effect of phosphate on the copper-amino acid complex has been reported(2). In the present studies it was found that the use of 0.5M phosphate buffer produced a non-linear relationship between lysine concentration and optical density. However, at a concentration of 0.15M phosphate, no such effect was observed.

For the following experiments standard curves were prepared from lysine dissolved in pH 7.0, 0.1M phosphate buffer at concentrations of 0.042M to 0.00525 lysine. The highest concentration of lysine represented 100 per cent hydrolysis of the LEE used in the hydrolysis experiments reported below.

Results. Reproducibility of the method. Lysine standard curves at pH 6.5 (Table I) were determined 6 times during a period of 2 weeks using a standard 0.042M lysine solution. The same copper phosphate was used throughout the experiments since no instability of the suspension was observed. It is calculated that the 95% confidence limits for the concentration of lysine in an unknown sample using a single lysine standard curve and a single reading on the unknown sample are ± 3.5%, irrespective of the lysine concentration. This figure may be taken as the probable maximum limit of error, taking into account possible errors in reading the unknown sample and in determining the lysine standard curve. If the 6 standard curves are averaged and plotted as one, the error in determining the standard curve will be reduced to a negligible amount, leaving only the error in reading the unknown sample. In this case the 95% confidence limits were calculated to be ± 2.5%.

TABLE I. Lysine Standard Curves Used for Statistical Analysis.

Molarity of lysine solution	Colorimeter readings 1	2	3	4	5	6
.042	312	305	307	306	309	315
.021	152	157	150	149	151	153
.0105	72	79	73	71	73	76
.00525	36	41	35	33	34	37

Rate of spontaneous hydrolysis of LEE. A 0.042M solution of LEE dihydrochloride dissolved in pH 7.8 0.1M phosphate buffer to yield a solution of pH 7.0. During incubation at 37°, 2 ml aliquots were removed at the indicated intervals and analyzed. The lysine content of each aliquot was determined by comparison with a lysine standard curve and the per cent hydrolysis of LEE plotted against the incubation time. The rate of 'spontaneous' hydrolysis of LEE is shown in Fig. 2. The data demonstrate that some hydrolysis of the ester occurs under the experimental conditions in the absence of enzyme. The hydrolysis value at zero time suggests either that the LEE contains a small amount of free lysine or that the addition of buffer or copper phosphate causes the formation of free lysine.

Hydrolysis of LEE by Trypsin. A 5 mg% solution of trypsin was prepared by dissolving the enzyme in 0.0025 N hydrochloride acid. LEE dihydrochloride was dissolved in pH

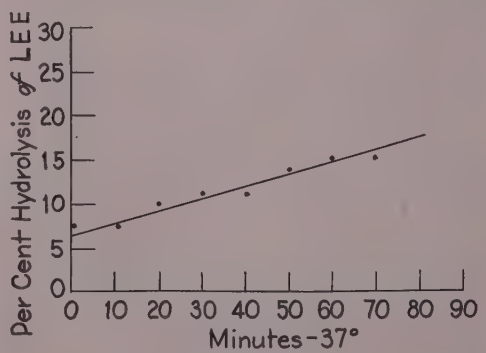

FIG. 2. Spontaneous hydrolysis of LEE at pH 7.

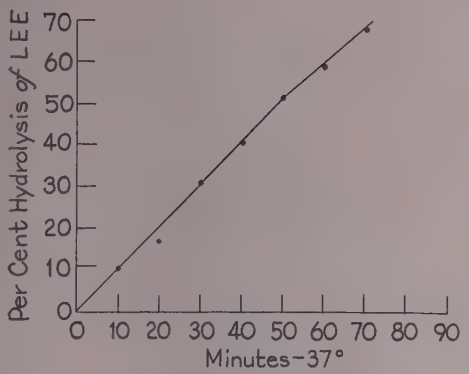

FIG. 3. Hydrolysis of LEE by trypsin at pH 7.

7.8, 0.4M sodium phosphate buffer to yield a 0.168M solution. The reagents were incubated separately at 37°C for 5 minutes with the exception of LEE which was prepared just prior to starting the test. The reaction mixture was prepared by combining 5 ml of enzyme solution, 10 ml of water and 5 ml of LEE solution. The substrate control contained 5 ml of 0.0025N HCl in place of the trypsin and the enzyme blank contained 5 ml of pH 7.0 0.4M phosphate buffer in place of the LEE. The final pH's of solution in all tubes was 7.0. After the addition of the LEE a timer was started and as incubation proceeded 2 ml aliquots were removed and analyzed. The colorimeter readings of the substrate control and enzyme blanks were subtracted from the colorimeter readings of reaction mixture and the corrected value was converted to per cent hydrolysis of LEE. The results are shown in Fig. 3. The data demonstrate that enzymatic breakdown of LEE may be conveniently followed by complexing the liberated lysine with copper.

The present studies employed trypsin as the lysine ethyl esterase but any lysine ethyl esterase may be used. The breakdown of LEE by plasmin (serum proteolytic enzyme) has also been studied by the above technic. Furthermore, by substituting the appropriate amino acid ester as substrate in the procedure described above, it has been possible to study the action of other amino acid esterases(4).

Summary. 1. The determination of lysine by reaction with copper phosphate has been described. 2. Within the range of concentrations studied the reproducibility of the method was found to be ± 3.5%. 3. The rate of hydrolysis of lysine ethyl ester by trypsin was determined by measurement of liberated lysine. 4. Application of the method to studies with lysine ethyl esterases and other amino-acid esterases was briefly discussed.

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Effect of Digitoxin and Quinidine on Intracellular Electrolytes of the Rabbit Heart.* (22566)

M. M. GERTLER,† J. KREAM, J. W. HYLIN, H. ROBINSON, AND E. G. NEIDLE.

Department of Cardiology, Francis Delafield Hospital, Columbia-Presbyterian Medical Center, N.Y.C.

Digitalis and quinidine preparations are much employed in the treatment of cardiac disorders(1). The beneficial effects of these drugs are ofttimes nullified by toxic phenomena which occasionally cause death(2,3). Although the exact mechanism for either toxicity or death is unknown, it has been suggested that electrolyte imbalance in the cardiac muscle results in establishing condi-

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† Present address: Co-ordinator of Medicine, Metropolitan Division, New York Medical College-Metropolitan Medical Center, N.Y.C.

tions which are favorable to the development of the toxic phenomena(4). The evidence for digitalis toxicity, albeit indirect, is fairly well established but far less is known concerning the concomitant electrolyte disturbances during quinidine therapy. In the main, studies concerning digitalis toxicity have established that there is a depletion of intracellular cardiac potassium with sequential changes of toxicity leading to death(4,5). Sherrod(6) has presented evidence which demonstrates potassium loss, sodium gain and calcium gain in following the use of digitalis dog ventricular muscle. Aikawa and Rhoades(7) have shown that there is a cellular depletion of potassium in rabbit atrium and ventricle following a single dose injection of digitoxin, 0.2 mg per kg. This loss in intracellular potassium is not only limited to cardiac muscle but is also observed in rabbit skeletal muscle (7) and has been known to reproduce the symptoms of familial idiopathic paralysis in humans(8).

Materials and methods. Seventy-four male albino rabbits weighing from 2650 to 3400 g were divided into 4 groups: (1) Eleven as a control group for quinidine treated animals, (2) thirty-two animals as a quinidine gluconate treated group, (3) ten as a control group for digitaline animals and (4) twenty-one as a digitaline nativelle treated group. The quinidine control animals received 1.5 ml quinidine gluconate solvent intramuscularly while the quinidine treated animals received the same volume which contained 0.12 g of quinidine gluconate.‡ The quinidine animals, both control and treated, were injected twice daily for 5 days and were sacrificed one hour after the last injection on the morning of the sixth day. The digitaline nativelle control animals were each injected with 1.5 ml of solvent and the digitaline nativelle treated animals received 1.5 ml of solvent containing 0.3 mg digitaline nativelle. Each animal received a daily intramuscular injection for 10 days and was sacrificed on the morning of the tenth day 2 hours after the digitaline nativelle injection.§

Blood was drawn from each rabbit's ear into a tube which contained 0.1 ml heparin as an anti-coagulant. The heart was removed and the ventricle was dissected at the atrioventricular groove; the ventricle was opened, the fat was trimmed and the valves were removed. The entire ventricle was blotted free from blood. One aliquot was removed for nitrogen determination while the remainder was placed in a tared weighing bottle and put into a vacuum oven at 60°C for 24 hours and reheated for 24 hours until constant weight was established. Skeletal muscle tissue was treated similarly to the ventricular muscle. After constant dry weight was established, the fat content was determined according to the method of Hastings and Eichelberger(9). The extracted fat was returned to the tube and the solvents were evaporated. The contents of the tube were digested in a boiling water bath in a solution containing 3.0 ml of concentrated nitric acid to which silver nitrate, 50% in excess of anticipated chlorides, was added. Chlorides were determined by the standard technic(10) after the solution was brought to known volume. Sodium and potassium were determined on two 1 ml aliquots of the digested solution in an internally standardized flame photometer after volume adjustments and lithium sulfate was added in the proper amount.

Derived values. The basic principle of Hastings and Eichelberger was used throughout(9). Serum water was determined by correcting serum concentrations after drying to a constant weight. Extra-cellular water (E.C.W.) was calculated as chloride space using a Gibbs-Donnan factor of 95 for striated muscle and 98 for heart muscle. In addition, the striated muscle and heart muscle were considered to contain 1% and 5% solids respectively. Intracellular water (I.C.W.) was obtained by subtracting the E.C.W. values from the tissue water values. Electro-

‡ Kindly furnished by Eli Lilly and Co., Indiana.

§ Kindly furnished by Varick Pharmacal Co., New York City. Solvent system contains:

	cc
Polyethylene glycol 300	28.6
Glycerin	43.8
Benzyl alcohol	4.
Ethyl alcohol	5.
Water for injection Q.S. A.D.	100.

TABLE I. Water, Electrolyte and Fat Values, Both Determined and Calculated, in Heart, Muscle and Serum in Normal Rabbits and Rabbits Treated with Digitaline Nativelle and Quinidine Gluconate.

				Muscle composition					Serum values				Calculated values		
				Cl	Na	K	H_2O,	Fat,	Cl	Na	K	H_2O	E.C.W. I.C.W.	Na	K
				mEq per kg fat free tissue			g % fresh tissue		mEq/l			%	ml/kg fat free tissue	mEq/l of I.C.W.	
Tissue	Sample	Drug	No.												
Muscle	Control		11	16.0	22.8	93.7	768	1.05	126	149	4.5	92.5	114 646	9.1	143
	Exp.	Quinidine	31	16.4	23.4	94.0	768	.74	122	159	4.7	92.8	119 649	5.9	144
"	Control		11	16.8	25.8	94.9	772	.53	125	157	4.8	92.9	119 653	10.8	144
	Exp.	Digitoxin	21	15.0	23.1	94.4	767	.75	136	153	5.3	92.9	106 661	10.4	142
Heart	Control		11	20.7	31.3	68.5	772	1.69	126	149	4.5	92.5	147 625	13.6	108
	Exp.	Quinidine	32	22.3	31.3	76.2	778	1.23	122	159	4.7	92.8	162 616	6.8	124
"	Control		10	20.9	32.0	67.5	789	.26	125	157	4.8	92.9	149 640	12.0	106
	Exp.	Digitoxin	19	20.4	32.7	70.2	775	.96	136	153	5.3	92.9	134 641	17.5	109

lytes were expressed in terms of a liter of I.C.W.

Results. The results of the experiments are summarized in Table I. The single important result here is a statistically significant increase in ventricular muscle of the intracellular potassium following quinidine injection from 106 ± 3.8 to 124 ± 2.1 (mean ± standard error) mEq per liter of intracellular fluids. There is also a statistically significant decrease in ventricular muscle of intracellular sodium in the same animals from 13.6 ± .9 to 6.8 ± .4 mEq per liter of intracellular fluids.

Discussion. The rabbits receiving digitaline nativelle intramuscularly did not evidence a potassium depletion as has been reported by other investigators(7). There is no obvious explanation for this observation except perhaps that the dose of digitoxin which was employed in this study was far less than that employed in the other studies mentioned. (Aikawa and Rhoades(7) used 0.2 mg/kg of rabbit in contrast to 0.1 mg/kg daily for 10 days in this study.) The dose of quinidine employed here was considered large in spite of the lack of comparisons.

There are several interesting implications of this study. It has been observed that the electrocardiographic signs of quinidine toxicity, *i.e.* sinus arrest, incomplete heart block, complete heart block and intraventricular block(11), are also seen in the early and terminal stages of uremia(12,13). It appears from this study and the other studies(12) that quinidine toxicity and uremia have a common denominator in that there is intracellular potassium retention in the heart ventricular muscle. One must not, however, infer from these observations that intracellular hyperpotassemia is the sole cause of these changes in both instances. The hypothesis that a decrease in intracellular cardiac potassium is accompanied by, and not necessarily causally related to, ventricular arrhythmias, and that an increase in intracellular cardiac potassium is accompanied by, and again not necessarily causally related to, a depression of cardiac arrhythmias is further strengthened from the observations of Harris *et al.*(4).

It is reasonable to suggest a possible mechanism of quinidine action in converting cardiac arrhythmias. It is now generally accepted that digitaline nativelle causes a depletion of intracellular cardiac potassium, and alters the concentration of other intracellular electrolytes such as sodium and calcium(6). The results of this study reveal that intramuscular quinidine gluconate produces in the rabbit intracellular hyperpotassemia, and possibly intracellular hyponatremia. (Other electrolytes such as Mg, Ca and Mn were not studied.) Therefore, quinidine gluconate may effect its action via intracellular enzyme systems which are either stimulated or inhibited by alteration in sodium, potassium or other trace metals. This possibility is being studied actively at present.

Conclusions. *1.* 32 rabbits were injected intramuscularly in the gluteal region with 0.12 g of quinidine gluconate twice daily and 21 rabbits were similarly injected daily with 0.3 mg digitaline nativelle for 5 days and 10 days respectively. *2.* A gain in intracellular cardiac potassium as expressed in liters of intracellular water was observed in the quinidine treated animals (from 108 to 124 mEq). A loss in intracellular cardiac sodium from 13.6 to 6.8 was also observed. *3.* The mechanism of digitoxin toxicity and its reversal by quinidine is discussed. It is suggested that one of the actions of quinidine may be to reverse the ionic flow of sodium and potassium (and perhaps other ions) in cardiac muscle which occurs in digitoxin toxicity.

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Oxidation of Specifically Labeled Glucose by Rat Adipose Tissue.* (22567)

S. W. MILSTEIN. (Introduced by Jay S. Roth.)

William Goldman Isotope Laboratory, Division of Biological Chemistry, Hahnemann Medical College, Philadelphia, Pa.

Recent studies have indicated that the rate of glucose utilization *in vitro* by adipose tissue of the rat is greater than that of liver(1,2). The extensive lipogenic activity of adipose tissue implies the occurrence of rapid glycolysis(3), but does not exclude other pathways of hexose metabolism. Considerable evidence for the extraglycolytic breakdown of glucose in hepatic and other tissues is available, atlhough accurate quantitation of its role in carbohydrate metabolism has been hampered by both technical and interpretative difficulties(4). Using one of the more direct experimental approaches to this problem (5), the oxidation of C^{14} glucose substrates, labeled in the first and sixth positions, has been studied in liver and testicular adipose

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TABLE I. Oxidation of Glucose-1-C^{14} and Glucose-6-C^{14} by Adipose Tissue and Liver. From 200 to 600 mg of tissue were incubated for 3 hr in 6.0 ml of buffer containing about 1 μc of the appropriate substrate.

Condition of animals			Adipose tissue			Liver		
			$C^{14}O_2$ yield* from		C-6†	$C^{14}O_2$ yield* from		C-6†
			G-1-C^{14}	G-6-C^{14}	C-1	G-1-C^{14}	G-6-C^{14}	C-1
Normal:	(2)‡		.72	.10	.14	.37	.20	.54
	(3)		.63	.07	.11	.29	.18	.62
	(2)		.53	.11	.21	.27	.23	.85
	(1)		.45	.12	.27	.40	.28	.70
Fasted:	(1)	18 hr	.27	.10	.37	—	—	—
	(1)	18	.27	.11	.41	—	—	—
	(1)	18	.27	.14	.52	—	—	—
	(3)	24	.16	.10	.63	.34	.27	.80
	(3)	48	.12	.08	.67	.27	.16	.60
	(3)	96	.19	.11	.58	.19	.09	.47
	(3)	192	.20	.11	.55	.16	.10	.63
Diabetic§:	(1)		.30	.11	.37	—	—	—
	(1)		.22	.12	.55	—	—	—
	(1)		.20	.10	.50	.10	.07	.70
	(2)		.11	.07	.63	.06	.04	.67
	(1)		.08	.04	.50	—	—	—
Cortisone-treated:								
$\frac{\text{mg/kg}}{\text{day}} \times \text{days}$		30 × 2	.22	.02	.11	.18	.14	.78
		42 × 6	.15	.11	.73	.41	.25	.61
		40 × 8	.21	.08	.40	.46	.28	.61
Insulin-treated diabetics:								
$\frac{\text{Units}\|}{\text{Doses}}$	Hr¶	BS**						
198/1	3.5	35	.18	.08	.44	.22	.20	.91
170/1	3.5	67	.17	.06	.35	.15	.14	.93
100/2	18	57	.96	.09	.09	.41	.31	.76
190/3	42	41	.92	.05	.05	.84	.34	.40
177/4	75	61	2.20	.20	.09	—	—	—

* Radiochemical yield $= \frac{\text{counts recovered as } CO_2/100 \text{ mg} \times 100}{\text{counts in substrate}}$. † $\frac{C^{14}O_2 \text{ from glucose-6-}C^{14}}{C^{14}O_2 \text{ from glucose-1-}C^{14}}$.

‡ No. of animals used in parentheses. Cortisone and insulin experiments done on individual rats. § Listed in order of increasing severity of diabetes. Lowest non-fasting blood sugar at sacrifice was 185 mg %, highest was greater than 600 mg %. ‖ Total insulin units/kg given in No. of doses indicated. ¶ Time from first insulin inj. to sacrifice. ** Blood sugar determined at sacrifice.

tissues from normal, fasted and hormonally manipulated rats.

Materials and methods. Male Wistar rats weighing between 200 and 400 g were fed on a stock Purina Chow diet unless otherwise indicated. Diabetes was induced by the intraperitoneal injection of alloxan monohydrate in animals fasted 48 hours and was permitted to progress untreated for at least 3 weeks. Protamine zinc insulin and cortisone, when administered, were given subcutaneously; the last injection was given 3 to 4 hours before sacrifice. Blood glucose levels were determined by the method of Durham *et al.*(6). The rats were sacrificed by a sharp blow on the head, exsanguinated and the required tissues immediately removed to ice-cold saline. Tissues from 1 to 3 animals were pooled in each flask. Stock solutions of the substrates (glucose-1-C^{14} and glucose-6-C^{14} obtained from the National Bureau of Standards) were prepared in saline and adjusted so that a final concentration of 100 mg% resulted in each flask. The incubations were carried out at 37°C as previously described(1). Radioactivity of the evolved carbon dioxide was determined according to Entenman *et al.*(7).

Results. The results, summarized in Table

I. indicate that the oxidation of carbon 1 of exogenous glucose is much more rapid than that of carbon 6 in normal adipose tissue, giving an average C-6/C-1 ratio of 0.18. Liver slices from the same animals exhibit significantly higher ratios, averaging about 0.68. On a wet weight basis, the rate of C-1 oxidation in adipose tissue is higher than that found in liver and, as previously indicated(1), this superiority is substantially amplified when the large difference in protein content between the tissues is considered. Even C-6 oxidation in adipose tissue is much greater than in liver when specific activity is related to nitrogen content. Prolonged starvation depressed the rates of C-1 and C-6 oxidation slightly in the liver, while their ratios remained normal. In adipose tissue, however, C-1 oxidation was significantly lowered after a short fast, while C-6 oxidation remained remarkably constant throughout the starvation periods studied, resulting in a rise of the C-6/C-1 ratio. Insulin deficiency, whether achieved by alloxan diabetes or cortisone administration, did not alter the ratio from normal in the liver, although a pronounced drop in oxidative rates of both labeled positions occurred in the alloxanized preparations. Again, C-6 oxidation by diabetic adipose tissues was relatively normal in magnitude, while the rate of C-1 oxidation varied inversely with the severity of the disease. In all instances, a significant elevation of the ratio is evident. Prolonged Cortisone administration also elevated the ratio by depressing the rate of C-1 oxidation. Administration of a massive dose of insulin (sufficient to cause coma within 3 to 4 hours) to diabetic rats fasted 24 hours, brought the hepatic rate of C-6 oxidation back to normal but failed to do the same for the C-1 oxidation, permitting the ratio to approach unity. However, the C-1 and C-6 oxidation rates and their ratios in adipose tissue appear similar to those of the diabetics. Gradual administration of insulin restored the normal rates of C-1 and C-6 oxidation in the liver and increased the C-1 oxidation above normal in adipose tissue, giving a significant depression of the latter's ratio.

Discussion. Although precise estimation of the fraction of glucose catabolized via the direct oxidative scheme in adipose tissue cannot be made from the data presented, it is obvious that under normal conditions it constitutes the major pathway of utilization. The shunt in adipose tissue appears to be very sensitive to starvation and the endocrine stresses which were imposed while glycolysis, as measured by the rate of C-6 oxidation, remained remarkably constant throughout. As a result, fasting, alloxan diabetes and cortisone treatment have the general tendency to reduce the total amount of glucose oxidized and permit glycolysis to account for a greater percentage of that which *is* catabolized. Insulin treatment of diabetics restored and enhanced both the total oxidative capacity of the tissue and the fractional importance of the shunt.

In the liver, the normal range of glycolytic participation in overall glucose metabolism was large enough to obscure small shifts in the quantitative significance of either pathway. While the total rates of oxidation seem to parallel the nutritionally and hormonally induced fluctuations in adipose tissue, no real change in the predominance of hepatic glycolysis is evident.

The significance of the relatively high rate of oxidation of glucose carbon 1 in normal adipose tissue may be intimately related to the extensive lipogenic capacity of that tissue. Lactating mammary gland tissue, in corelation with its stimulated fatty acid synthesis, has been shown to exhibit a low C-6/C-1 ratio similar to that of adipose tissue(8,9). Diabetic tissues usually demonstrate an elevated C-6/C-1 ratio(10) and a markedly decreased ability to synthesize fat. Hepatic lipogenic failure has been noted previously in insulin coma(11) and has been here shown to be associated with a C-6/C-1 ratio of practically 1. A tempting speculation arising from these and other correlations(12,13) is that lipogenesis is very sensitive to, or at least proceeds more readily with concomitant rapid oxidation of glucose carbon 1. While glycolysis appears to be a general prerequisite for lipogenesis(3), equally important reductive steps in the fatty acid cycle(14) must be considered in a complete evaluation of the proc-

ess. Two reactions of this cycle involve hydrogen transfers and the redox status of the enzymatic cofactors concerned may determine not only the extent but the direction (*i.e.*, synthesis or degradation) in which the cycle may turn. Recently, a specific requirement for triphosphopyridine nucleotide (TPN) in liver fatty acid synthesis has been demonstrated(15). If this system is operative in adipose tissue as well, then the availability of the reduced coenzyme (TPNH) would become a critical aspect of lipogenesis. The physiological appearance of TPNH, exclusive of the presence of transhydrogenase activity (16), is dependent on the activity of TPN-linked dehydrogenases, prominent among which are those involved in the phosphogluconate shunt. The presence of pyridine nucleotides (DPN and TPN) and their reduced forms in adipose tissue is currently under investigation.

Summary. The *in vitro* oxidation of glucose-1-C^{14} and glucose-6-C^{14} by liver and adipose tissues of the rat has been studied. 1) In adipose tissues from normal animals, the yield of $C^{14}O_2$ from glucose-6-C^{14} averaged about 18% of that obtained from glucose-1-C^{14}, indicating the extensive occurrence of extraglycolytic catabolism of glucose. Liver slices from these animals showed a corresponding $C^{14}O_2$ ratio of 68%. 2) Starvation (from 18 to 192 hours) depressed the $C^{14}O_2$ yield from glucose-1-C^{14} without affecting the yield from glucose-6-C^{14} in adipose tissues. Similar changes were seen in adipose tissues from alloxan diabetic and cortisone-treated animals. In all these cases, the C-6/C-1 ratio was significantly elevated above normal. In the liver, prolonged starvation (96 to 192 hours) lowered the $C^{14}O_2$ yields from both substrates without affecting their ratio. Marked depressions of $C^{14}O_2$ yields were seen in diabetic livers, but no changes in C-6/C-1 ratio were evident in these, as well as in livers from cortisone-treated rats. 3) Fasted diabetic rats responded to a massive insulin dose with typical symptoms of insulin coma. Adipose tissue oxidation rates of both substrates and their ratio remained characteristically diabetic while the liver ratio approached unity. Previous work indicates that hepatic lipogenesis is abolished under such conditions. Gradual administration of insulin to *ad lib.* fed diabetics resulted in a stimulation of $C^{14}O_2$ yield from glucose-1-C^{14} without affecting the yield from glucose-6-C^{14} in adipose tissue. Both $C^{14}O_2$ yields returned to normal levels in the liver with no change in ratio.

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Effect of Histamine, Hog Vasopressin, and Corticotropin-Releasing Factor (CRF) on ACTH Release *in vitro*. (22568)

A. V. Schally and Murray Saffran. (Introduced by T. L. Sourkes.)
Allan Memorial Institute of Psychiatry, McGill University, Montreal, P. Q.

Both vasopressin and histamine have been suggested as neurohumoral mediators in the release of ACTH(1-3). Recently, Swingle *et al.*(4) reported that an acid extract of pitressin contained a histamine-like substance and was active in releasing ACTH *in vitro*. Martini *et al.*(5) claimed that both oxytocin and vasopressin can induce a discharge of ACTH if injected into normal rats. However, Guillemin and Hearn(6) demonstrated that highly purified arginine (beef) vasopressin was without effect on the release of ACTH by tissue cultures of rat pituitaries. Our own work(7) has shown that corticotropin-releasing-factor (CRF) is distinct from oxytocin and beef vasopressin. This paper describes the effects of histamine, purified lysine (hog) vasopressin and purified CRF on the release of ACTH *in vitro*.

Materials and methods. Histamine dihydrochloride (Fisher Reagent); the amounts added are expressed as μg of histamine base per incubation flask. Vasopressin, from hog sources, was prepared according to the method of Stehle and Fraser(8) and subjected to paper chromatography in butanol:acetic acid: water by the procedure of Benfey*(9). This preparation, possessing a pressor activity of 200 U./mg, was assayed for CRF activity and then further purified by paper chromatography in m-cresol(10). Purified CRF was prepared from Protopituitrin† (Parke, Davis, and Co.), which corresponds to fraction C of Kamm(11). The purification procedure for CRF will be reported elsewhere(10). The detection of CRF activity was made according to the method of Saffran and Schally(12) using isolated rat pituitaries and the *in vitro* bioassay of ACTH by the method of Saffran and Schally(13). The results are expressed as the ratio of the ACTH released in the presence of the substance tested to the ACTH released by the control, accompanied by the 95% confidence limits. A significant stimulation of ACTH-release is denoted by a lower confidence limit greater than 1.0.

TABLE I. CRF and ACTH-Release.

CRF preparation	l-Arterenol .0002M	Dose CRF, μg/flask	Ratio, exp. to control	95% limits
Purified 40×	Present	.26	2.1	1.6→2.6
Idem	"	.26	2.15	1.3→3.5
"	Absent	.26	2.5	1.7→3.6
"	"	2.6	3.4	2.9→4.0
Purified 110×	"	.09	2.3	1.3→4.1

Results. Table I shows the results obtained with CRF preparations at various purification stages, with or without arterenol. Small doses of purified CRF significantly double the release of ACTH. This was the case whether arterenol was present or not. The resuts obtained with histamine are shown in Table II. Arterenol was omitted in tests with histamine. Histamine did not significantly increase the release of ACTH *in vitro*, whereas in one experiment a dose of 0.01 μg significantly inhibited the release of ACTH. Results obtained with hog vasopressin are shown in Table III. There was a slight, but not significant increase in the release of ACTH. After further purification in m-cresol, in which vasopressin and CRF have distinctly different R_f's, CRF activity in vasopressin was not detectable.

Discussion. Harris *et al.*(14) were able to extract histamine from hypothalamus and pos-

TABLE II. Histamine and ACTH-Release.

Dose, histamine/flask (μg)	Ratio of experimental to control	95% confidence limits
.01	.5	.4 → .8
.1	.5	.2 → 1.3
.1	1.1	.8 → 1.4
.5	1.5	.6 → 3.9

* We are indebted to Dr. B. G. Benfey for his gift of purified lysine vasopressin.

†We are grateful to Dr. D. A. McGinty of Parke, Davis, and Co. for generous gifts of posterior pituitary preparations.

TABLE III. Hog Vasopressin and ACTH-Release.

Preparation	l-Arterenol .0002M	Dose vasopressin per flask, μg	Ratio, exp. to control	95% limits
Benfey	Present	.5	1.5	.9→2.6
"	"	.5	1.4	.8→2.6
m-cresol purified	Absent	.2	1.3	.9→1.7
Idem	"	.5	.9	.8→1.2

terior pituitary tissue. The results reported here indicate that free histamine is not the neurohumour responsible for the release of ACTH. If histamine in pitressin is bound in a peptide linkage, as Swingle *et al.*(4) suggest, then it should have been liberated by their extraction procedure. In our hands posterior pituitary preparations lose their CRF activity when subjected to overnight hydrolysis in 6N HCl at 110°C(15), a procedure which would not destroy histamine. Similarly, we cannot support the claims of Martini *et al.*(5) that vasopressin may stimulate the anterior lobe to release ACTH. Their results were obtained with impure preparations which could contain CRF(7). The same conclusion, with respect to the absence of CRF activity in histamine, was reached independently by Guillemin(16).

Summary. Histamine and purified lysine (hog) vasopressin did not accelerate the release of ACTH by isolated rat pituitaries. In contrast, purified CRF preparations in minute doses significantly increased the release of ACTH.

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Sulfamethoxypyridazine: Preliminary Observations on Absorption and Excretion of a New, Long-Acting Antibacterial Sulfonamide.* (22569)

ROGER L. NICHOLS, WILFRED F. JONES, JR. AND MAXWELL FINLAND.
Thorndike Memorial Laboratory, Second and Fourth (Harvard) Medical Services, Boston City Hospital and the Department of Medicine, Harvard Medical School, Boston, Mass.

Antimicrobial agents that have a prolonged action have potential advantages, particularly if they are effective in prophylaxis, or in the prolonged treatment of chronic or subacute infections, or in the suppression of chronic infections that are difficult to eradicate. A new antibacterial sulfonamide, sulfamethoxypyridazine (3-sulfonamido-6-methoxypyridazine)† was found to have the following properties, judged from experiments in dogs, rabbits and mice: high solubility in urine, good absorp-

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† Generously provided by Lederle Laboratories Division, American Cyanamid Co. under the trademark Kynex (Lederle).

tion from the gastrointestinal tract, very slow urinary excretion, poor acetylation, good penetration into the brain, and antibacterial activity equivalent to sulfadiazine(1). These properties were of sufficient interest to warrant exploration of this agent in humans. Preliminary observations on absorption and excretion of this compound after a single oral dose in normal subjects are here presented.

Materials and methods. Each of 6 normal, young adult males was given 4.0 g (8 tablets of 0.5 g each) orally with a glass of water before breakfast. Oxalated venous samples of blood were obtained just before and 1, 3, 5, 8, 12, 24 and 105 hours after the dose. Each subject emptied his bladder just prior to the dose and complete collections of urine were made during the following 72 hours in 2, for 48 hours in 2 others and for 24 hours in a fifth subject. Food and fluids were taken *ad libitum*. A voided specimen of urine was also obtained from each subject at the time of the last blood. The concentrations of free and total sulfonamide, calculated as sulfamethoxypyridazine, were determined by the method of Bratton and Marshall(2). Part of some of the specimens of blood was also used to determine the hematocrit and the concentration of the drug in the plasma in order to calculate the concentration in the blood cells and the rate of renal clearance of the drug from the plasma.‡

Results. Blood levels. The concentration of total sulfamethoxypyridazine in the whole blood of each of the subjects is shown in Fig. 1; the averages of these values and of those for the free drug in all of the subjects are also shown. The maximum concentrations (average about 15 mg per 100 ml total and 13 mg per 100 ml free) were achieved 5 hours after the dose was taken and maintained for the next 3 hours; after that time the levels fell rather slowly, although the rate of fall was somewhat faster between 8 and 12 hours than it was later. At 24 hours the average concentrations of total and free drug were 12.1 and

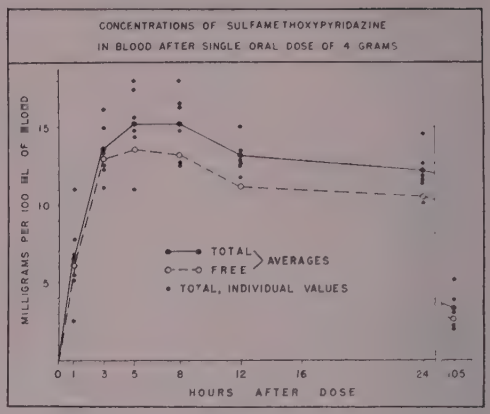

FIG. 1.

10.3 mg %, respectively. After 105 hours there were still appreciable concentrations in the blood, averaging 3.3 and 2.8 mg % total and free drug, respectively. The proportion of the drug in conjugated—presumably acetylated—form was very small in the first 3 hours and subsequently averaged less than 15% (range 5-22%). *Diffusion into blood cells.* The concentrations of total sulfamethoxypyridazine observed in whole blood and plasma and the concentrations in the cells calculated from these values and the hematocrit are shown for a few random specimens in Table I. These crude values indicate little or no diffusion of the drug into the blood cells. *Renal clearance.* The rates at which sulfamethoxypyridazine is cleared from the plasma

TABLE I. Diffusion of Sulfamethoxypyridazine into Erythrocytes.

Subject	Hr after dose	Hematocrit	Conc.,* mg/100 ml — Whole blood	Plasma	Erythrocytes, calculated
W.J.	5	48.3	11.1	25.4	0
	8	46.3	12.6	22.6	1.1
	12	47.0	11.8	24.4	0
	24	47.5	12.6	21.0	3.5
	105	47.5	4.1	8.9	0
R.N.	5	42.5	14.8	25.2	0.7
	8	42.6	12.6	22.4	0
	12	41.6	12.7	25.2	0
C.P.	8	44.7	16.4	28.2	1.8
R.C.	3	46.9	15.0	23.6	5.3
	5	44.7	15.6	29.6	0
	8	43.6	16.4	26.2	3.7

* Total drug.

‡ The authors are indebted to Miss Ellen J. Doyle and Miss Mary I. Kendrick for carrying out the chemical determinations.

TABLE II. Renal Clearance of Sulfamethoxypyridazine.

Subject	Period of study: Hr after dose	No. of min.	Avg concentration in plasma, mg/100 ml: Free	Acetyl	Amt excreted in urine during period, mg: Free	Acetyl	Plasma cleared, ml/min.: Free	Acetyl	Clearance ratio: Acetyl/Free
W.J.	5- 8	180	22.2	1.8	49	34	1.2	10.5	8.6
	8-12	240	21.8	1.7	64	52	1.2	12.8	10.7
R.N.	5- 8	180	22.8	1.0	79	49	1.9	27.0	14.2
	8-12	240	22.8	1.0	147	79	2.7	33.0	12.2
C.P.	5- 8	180	23.0	5.2	78	146	1.9	15.6	8.2

by the kidney were calculated in 3 subjects for periods when the blood levels were at or near maximal (Table II). The free drug was cleared at a very slow rate, averaging 1.8 ml of plasma per minute; the acetylated drug was cleared at an average rate of 19.8 ml per minute or 11 times that of the free drug. *Urinary excretion.* Since the fluid intake was not controlled in this study, the rate of urine flow varied considerably among the subjects and at different times in the same subject. As a result, the concentrations of drug in the urine varied widely (Fig. 2). In the subject with the largest volume of urine concentrations of total drug in the individual specimens of urine collected between 5 and 24 hours after the dose varied between 29 and

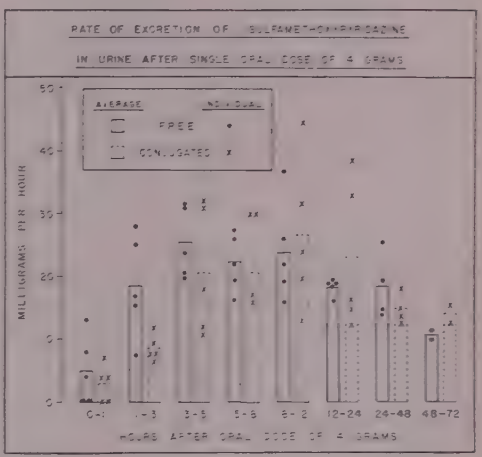

FIG. 3.

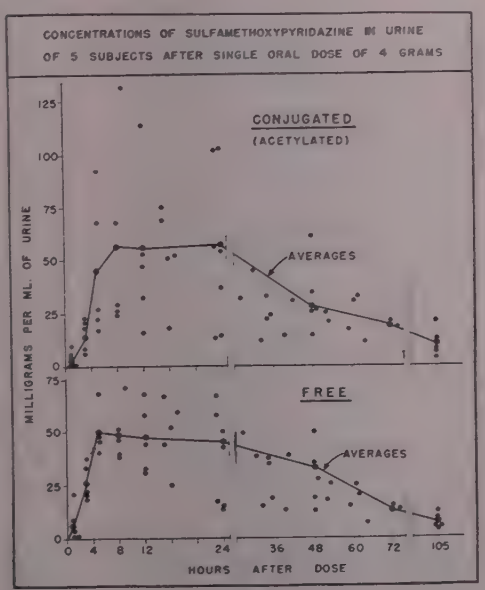

FIG. 2.

66 mg %, in contrast to values ranging from 136 to 204 mg % in the subject who put out the smallest amount of urine. The *rate* of excretion of the drug, particularly in the free form, showed much less variation (Fig. 3). The volume of urine in any given period did not seem to have any marked effect on the rate of excretion of the drug. Average curves for the cumulative excretion of the drug over 72 hours are shown in Fig. 4. In the first 24 hours the average amount excreted was 24.6% (range 18.8 to 31.0%) of the administered dose; an additional 20.2% (range 17.7 to 26.3%) was excreted from 24-48 hours and, of the 2 subjects whose urine was collected during the third 24-hour period, one excreted 13.6% and the other 12.6% of the dose in that time. Of the amount excreted, the proportion determined to be in the conjugated form at different times varied from 35 to 60%, similar fluctuations being noted in each of the sub-

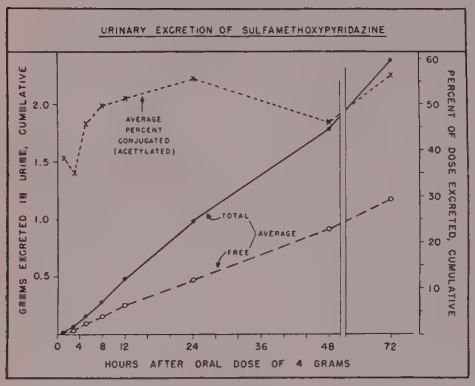

FIG. 4.

jects. Specimens of urine collected 105 hours after the dose still contained concentrations of drug averaging 19.3 mg % total and 7.8 mg % free.

Untoward effects. The urinary sediment was examined microscopically in specimens collected between 8 and 24 hours and revealed no abnormalities. The only untoward effect experienced by 3 of the 6 subjects was some lassitude followed by frontal headache that began between 3 and 5 hours after the dose and lasted 4 or 5 hours; the headache was aggravated by sudden motions of the head but this did not interfere with normal activity.

Summary and conclusions. Concentrations of sulfamethoxypyridazine in blood and urine of 6 normal adult males were determined after a single oral dose of 4 g. The drug was well absorbed, yielding high levels of free drug and only small amounts in acetylated form in the plasma. Little if any of the drug diffuses into the blood cells. The drug is cleared slowly from the plasma, the acetylated form being cleared by the kidney about 11 times as fast as the free drug. Urine concentrations varied up to about 200 mg %, between 35 and 60% being in the conjugated form. Significant levels were still present in the blood and urine 105 hours after the dose. This prolonged action should be of clinical interest and suggests that further exploration of the potentialities of this new sulfonamide drug is warranted.

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Morphological Transformation of *Candida albicans* in Tissues of Mice.* (22570)

DOUGLAS W. HILL AND LOUIS P. GEBHARDT.
Department of Bacteriology, University of Utah College of Medicine, Salt Lake City.

Organisms of the genus *Candida* exhibiting filamentous morphology are commonly seen in smears prepared from the oral and vaginal cavities of infected persons. Cells of *C. albicans* with filamentous morphology have also been observed in deeper tissues including nervous tissue, kidneys, adrenals, cardiac and skeletal muscle and various portions of the gastrointestinal tract(1-5).

In rabbits experimentaly infected by the intravenous injection of *C. albicans*, large numbers of filamentous cells are found especially in kidneys(6,7). On the basis of morphology in tissue Baker(8) has classified *C. albicans* with those fungi which are found in tissue as "filaments and round bodies." Scherr and Weaver(9) have speculated that "when the mycelial phase of the fungus becomes evident in the later stages of an infection, it would appear that some factor which normally favors the Y (yeast) forms has diminished in function."

While studying the host tissue reaction to washed yeast-phase cells of pathogenic fungi, it was observed that cells of *C. albicans* rap-

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idly underwent morphological alterations after injection into the subcutaneous tissues of mice. The nonpathogenic members of the genus *Candida* studied did not exhibit this change. The morphological alteration of *C. albicans* was so rapid as to suggest a possible role in the pathogenesis of experimental moniliasis in the mouse.

Materials and methods. Six different strains of *C. albicans*, all virulent for mice, and one strain each of *C. stellatoidea, C. tropicalis, C. pseudotropicalis, C. krusei, C. parapsilosis* and *C. guilliermondi* were used in this study. Two of the strains of *C. albicans* were of recent origin, one isolated from the oral cavity of a 5-year-old child with a clinical diagnosis of oral thrush and the other from the throat of a week-old infant exhibiting white, membranous plaques in the oral cavity. The remaining strains of *C. albicans* had been maintained on Sabouraud medium for undetermined periods of time. All strains of *C. albicans* and the single strain of *C. stellatoidea* readily produced chlamydospores on the glycogen medium described by Nickerson and Mankowski(10).

The organisms were maintained on Sabouraud-maltose agar with yeast-extract (0.01%). All of the strains grew in the typical smooth, yeast-like phase on this medium. The inoculum for injection into mice consisted of organisms washed with 0.85% sodium chloride solution from 48 hour agar cultures incubated at 35°C. The cells were washed 2 times by centrifugation and resuspended in fresh saline solution. No attempt was made to standardize the number of organisms contained in the suspensions other than by visual approximation.

Adult albino mice were used in these experiments. Cells of members of the genus *Candida* were injected into the subcutaneous tissues of these animals and the inflamed areas were studied at various time intervals after injection. One-tenth ml of the above suspensions of cells was injected into the subcutis of the animal's abdomen. At different time intervals mice were sacrificed and thin sheets of loose connective tissue were excised from within the area of injection. These sheets of areolar tissue were rapidly spread on microscope slides, air-dried and stained by the May-Grünwald-Giemsa method. The yeast cells stained a light blue by this procedure which made them easily distinguishable from host cells. This technic was essentially that used by Schneebeli and Dougherty(11) to study early inflammation in the mouse.

A second method similar to the first was used later because of greater ease in preparing the connective tissue spreads(12). It was a modification of the "granuloma pouch" technic of Selye(13). One ml of air was injected subcutaneously into the nape of the neck of mice. Subsequently the organisms contained in 0.2 ml of diluent were injected into the formed air pouch. At different times after inoculation animals were sacrificed and the intact air pouch partially dissected free. Sheets of connective tissue comprising the wall of the base of the pouch were removed and treated as previously described.

Results. A striking morphological alteration occurred in the cells of all of the strains of *C. albicans* studied. Within 60 minutes after injection into the subcutaneous tissues of mice almost all of the yeast-like cells of *C. albicans* had formed a short rudimentary pseudomycelium (Fig. 1a). Cells suspended in the saline diluent retained their typical yeast-like morphology. Various stages in development of the pseudomycelia were observed. Slides prepared 2 hours after injection revealed an increase in length of the pseudomycelia with beginning septa formation (Fig. 1b). Four hours after injection definite septa were evident (Fig. 1c). With the exception of *C. stellatoidea* these *in vivo* alterations in morphology were not observed when other species of *Candida* were injected. These organisms retained their typical, yeast-like morphology (*e.g.* Fig. 1e and 1f). The single strain of *C. stellatoidea* which was studied exhibited *in vivo* pseudomycelial formation similar to that shown by *C. albicans* strains.

At 6, 12 and 24 hours after injection of yeast-like cells of *C. albicans* and *C. stellatoidea* typical pseudomycelia were observed in spreads prepared from the loose connective tissues of mice. In the case of *C. albicans* some were observed which were several oil-

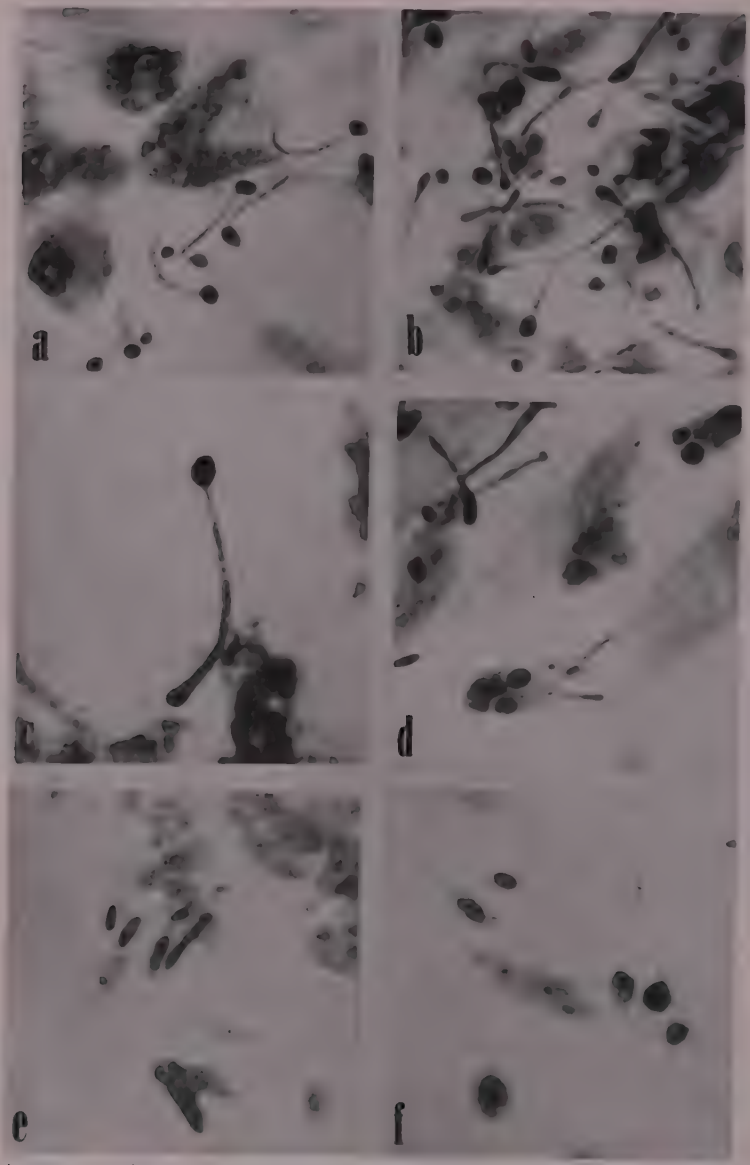

FIG. 1. Appearance of cells of *C. albicans* and other *Candida* species after inj. of washed yeast-like cells into the subcut. tissues of mice. a. *C. albicans* 1 hr after inj. b. *C. albicans* 2 hr after inj. c. *C. albicans* 4 hr after inj. d. *C. albicans* cells 2 hr after inj. demonstrating ingestion by phagocytes. e. *C. krusei* 4 hr after inj. f. *C. tropicalis* 4 hr after inj.

immersion fields in length. Lateral blastospores were observed. As judged by their staining characteristics, however, the majority of the organisms observed 24 hours after injection appeared to be degenerating. In the case of other species of *Candida* studied it was difficult to demonstrate organisms in the subcutaneous connective tissues of mice at this latter time. Phagocytosis of yeast-like cells was observed in subcutaneous tissues taken from mice injected with all species of *Candida* studied. The ingested cells exhibited

various stages of digestion as evidenced by loss of the ability to take the basophilic stain. With preparations from mice injected with cells of *C. albicans* rarely was a phagocyte observed which had ingested an organism showing any degree of pseudomycelium formation (Fig. 1d). Occasionally a phagocyte was seen which had ingested the "head" portion with the "tail" projecting outward.

Discussion. After completion of these observations the work of Stovall and Pessin(7) was found in the literature. They studied the behavior of injected cells of *"Monilia albicans"* (*C. albicans*), *"M. candida"* (*C. tropicalis*) and *"M. parapsilosis"* (*C. parapsilosis*) in rabbits. It was reported that only the cells of *"M. albicans"* would form pseudomycelia *in vivo*. The authors suggested that *"M. albicans"* produced lesions by a "purely mechanical plugging of capillaries and arterioles." However, on the basis of measurements of cells grown for 48 hours on malt agar, they were unable to find a correlation between cell size and pathogenicity of the 3 organisms studied. The principal quality which accounted for the virulence of *"M. albicans"* was suggested to be the ability to grow in the animal body. It is apparent that the size of yeast-like cells of *C. albicans* grown on malt agar has little relationship to the size of the filamentous cells *in vivo*. The findings reported here partially substantiate the earlier work of Stovall and Pessin(7). Of the organisms studied in this laboratory, only *C. albicans* and *C. stellatoidea* exhibited filamentous morphology in the tissues of the mouse. All species of *Candida* usually considered non-pathogenic failed to develop pseudomycelia *in vivo*. The fact that the strain of *C. stellatoidea* behaved similarly to the strains of *C. albicans* would appear to indicate their close relationship. The rapidity of the alterations in morphology of *C. albicans* in the connective tissues of the mouse would seem to indicate a significant role for this alteration in the pathogenesis of experimental moniliasis. It would appear reasonable to suggest that this transformation favors survival of the fungus in the host by a mechanical interference of ingestion by phagocytes and by possible "mechanical plugging of capillaries and arterioles." On the basis of the speculation that the yeast-like form of *C. albicans* is best adapted to development in tissues, Scherr (14) postulated that any agent which would transform the organism from the yeast-like phase to the mycelial phase *in vivo* might arrest the multiplication of the pathogen. To examine this hypothesis he tried a number of compounds with plant growth-promoting activity. These compounds were given to mice systemically infected with *C. albicans*. The results of these experiments were negative. The fact that large numbers of filamentous organisms are found in the tissues of infected mice and rabbits may indicate that exactly the opposite situation may exist, *i.e.*, that the filamentous form of *C. albicans* is best adapted to growth in animal tissues. Experiments designed to test this suggestion are in progress. Factors affecting the morphology of *C. albicans in vivo* are also under investigation.

Summary. A morphological alteration of yeast-like cells of *C. albicans* and *C. stellatoidea* has been shown to occur within 1 hour after injection into the subcutaneous tissues of mice. Yeast-like cells of other members of the genus *Candida* failed to exhibit these alterations under the same conditions. The yeast-like cells of *C. albicans* had formed elongated pseudomycelia within 1 hour after injection. At later times considerable growth of these filaments with the appearance of septa was observed. Other species of the genus *Candida* retained their typical yeast-like morphology. It has been postulated that filamentation of *C. albicans in vivo* serves as a hindrance to ingestion by mouse phagocytes. The results suggest a significant role for these morphologically altered organisms in the pathogenesis of experimental moniliasis in the mouse.

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Pathophysiology of Malignancy: I. Tissue Oxygen Tension of Benign and Malignant Tumors of the Skin.* (22571)

F. URBACH.

Departments of Dermatology, Roswell Park Memorial Institute, and Physiology, RPMI Division, Graduate School of Arts and Sciences, University of Buffalo, N. Y.

Many facets of tumor metabolism have been investigated, leading to the conclusion that demonstrable alterations of aerobic and anaerobic respiration exist in malignant cells (1,2). Yet little attention has been paid to the physiologic aspects of oxygen transport and supply in tumor tissue. Thus it seemed appropriate to investigate the parameters of tumor tissue oxygen tension, and study the mechanisms of its control.

In the past, tissue oxygen tension has been mainly estimated from the oxygen content of arterial and venous blood. This method, limited to organs whose rate of capillary circulation can be measured accurately, leads only to gross estimates of tissue oxygen tension changes, and is generally unsuitable for most malignant tumors because of the difficulty of obtaining representative samples of venous blood. Before the introduction of the platinum oxygen cathode, the nearest approach to a satisfactory method for measuring oxygen tension in intact tissues was the one in which gas bubbles, introduced into a tissue, were allowed to come to equilibrium in respect to oxygen, and the gas then withdrawn and analyzed(3). This method is prone to large errors due to compression of surrounding vessels, it cannot measure rapid changes of pO_2 nor is it applicable to estimation of oxygen tension of small volumes of tissue.

The platinum oxygen cathode, described by Davies and Brink(4), has been used extensively for rapid estimation of oxygen tension in intact skin(5), heart(6) and muscle(7). A description of the basic instrumentation and the physiologic variables applicable to its use in skin has been given by Montgomery and Horvitz(5).

Method. Cutaneous oxygen tension was measured *in vivo* by means of the open type oxygen cathode(5). The diameter of the exposed platinum surface was approximately 200 μ. In order to minimize variations in blood flow, all measurements were performed in a warm room (temperature 28° to 30°C) and comparative pO_2 determinations of abnormal and adjacent normal skin were performed simultaneously. The electrodes used were calibrated against each other in saline (equilibrated against air) to be certain that the platinum surfaces exposed were comparable. Three or more consecutive values (obtained by re-insertion of the electrodes into adjacent tumor and normal skin sites respectively) were averaged and compared to each

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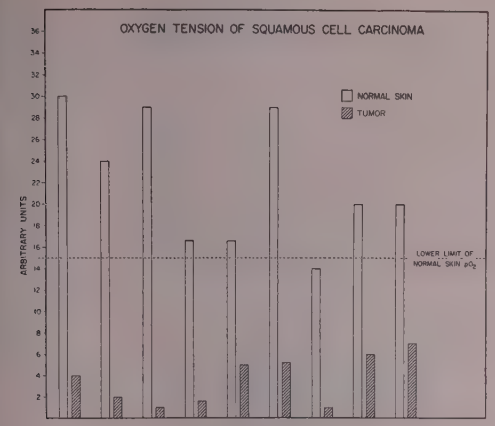

FIG. 1.

other. Biopsies were taken from the areas examined to rule out the possibility of local necrosis at the electrode insertion sites. Each of a pair of electrodes was used alternately in normal and in abnormal skin, so as to further rule out any fixed inherent error.

Apparatus. Platinum microelectrodes were made according to the technic described by Montgomery and Horvitz(5), but using 32 gauge, 90% platinum-10% iridium wire. A silver-silver chloride indifferent electrode was used to complete the circuit. All readings were taken with an applied DC voltage of —0.6 volt, and the current obtained from the electrode system amplified by a specially designed push-pull DC amplifier and fed into a Speedomax x_1-x_2 recorder. Skin temperature was measured simultaneously in some experiments by means of a thermistor needle (inserted into the experimental site) driving the x_2 pen of the recorder. All results were expressed in terms of arbitrary units, each unit corresponding to a current of 3×10^{-11} ampere.

Technic. Patients were placed on a bed in a constant temperature room kept between 28° and 30°C. Electrodes were inserted into the lesions to be studied and into the normal appearing, immediately adjacent skin. The circuit was completed by a finger dipped into a beaker of saline containing the silver-silver chloride anode. Three pO_2 readings were taken at each electrode insertion after equilibrium had been established (denoted by stabilization of electrode current). The electrodes were then re-inserted and the procedure repeated.

Results. 1. *Oxygen tension of normal skin.* In over 600 individual measurements of the oxygen tension of the normal skin of more than 200 patients, it was found that, provided good blood flow was present, electrode currents exceeded 15 units (4.5×10^{-10} amp.). While there existed marked individual and

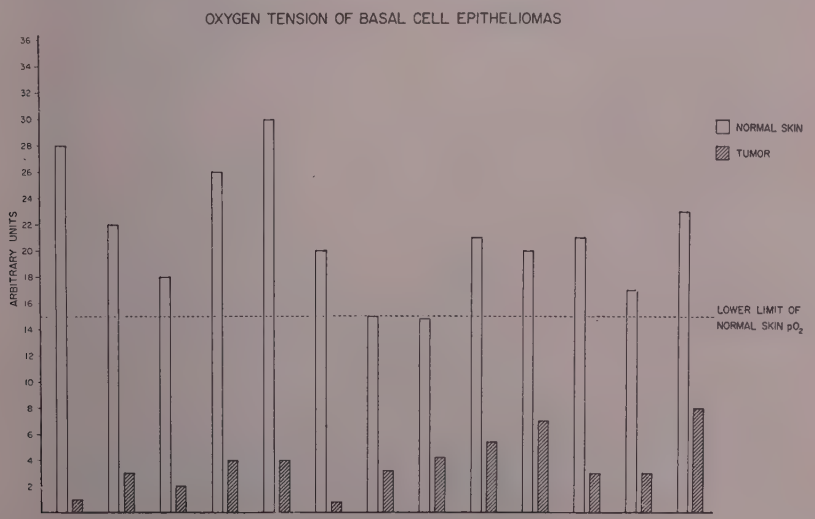

FIG. 2.

daily variations, sometimes approaching 50%, consecutive pO_2 measurements with matched electrodes in any one skin area under closely similar conditions of temperature and blood flow varied no more than 20%.

2. *Oxygen tension of malignant cutaneous tumors.* Eight different varieties of malignant tumors were examined: 9 squamous cell carcinomas, 13 basal cell epitheliomas, 6 metastatic cutaneous melanoma lesions, 2 carcinomas of the breast involving the skin, 2 lesions of dermatofibrosarcoma and 1 tumor lesion each of mycosis fungoides and reticulum cell sarcoma. All bled easily on insertion of the electrodes. In all cases, biopsies obtained from the site of electrode insertion proved the absence of necrosis and the identity of the tumor. In almost all of these malignant lesions, the oxygen tension within the tumor mass was found to be 25% or less of that of the simultaneously examined normal adjacent skin (Fig. 1-3). In several cases, it was possible to outline the extent of tumor infiltration quite accurately, using the low pO_2 as indicator of the extent of involvement.

3. *Oxygen tension of cutaneous lesions characterized by dense cellular infiltrate or acanthosis.* Five dermal cellular nevi on 4 patients, 4 xanthoma tuberosum nodules on 3 patients, 3 sarcoid granulomata on one patient, 2 patches of thickened psoriasis, 3 lichen planus papules on one patient and 2 verruca vulgaris on 2 patients were examined. With the exception of 2 of the nevi, 1 xanthoma tumor and the 2 verrucae, the remaining lesions showed a pO_2 of less than 25% of that of the adjacent normal skin (Fig. 4, 5). All lesions except the nevi and warts bled easily on electrode insertion. In all lesions, dense cellular infiltrate consisting

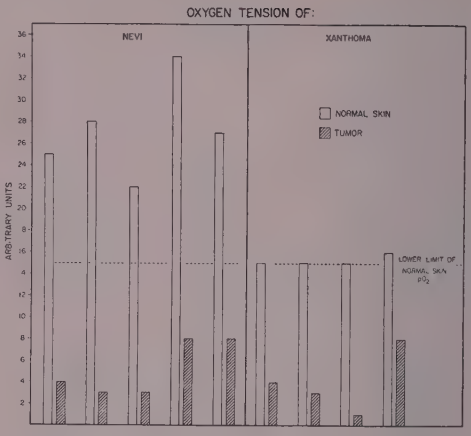

FIG. 4.

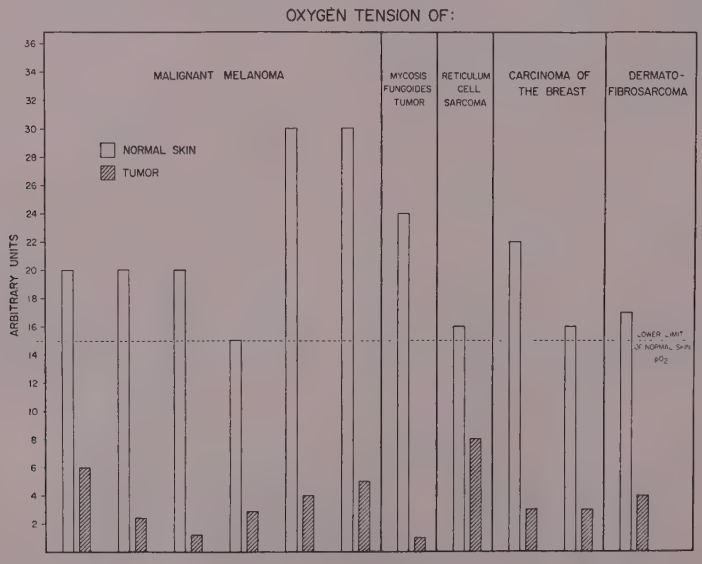

FIG. 3.

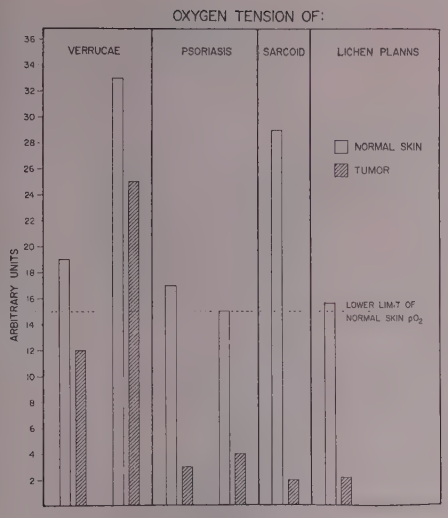

FIG. 5.

of either nevus cells, foam cells or inflammatory cells was noted on biopsy. The psoriatic patches and verrucae showed acanthosis as well as marked round cell infiltration.

4. *Oxygen tension of cutaneous lesions characterized by relatively minor cellular infiltrate and little or no acanthosis.* Six seborrheic keratoses on 4 patients, 8 arsenical keratoses on 2 patients, 1 stasis ulcer, 3 x-ray scars on 3 patients, 1 area of localized pretibial post-hyperthyroid myxedema of the skin and 4 areas of one case of premycosis fungoides were examined. With the exception of 2 arsenical keratoses (where biopsy showed dyskeratosis and early metaplasia) and the x-ray scars (which appeared histologically markedly avascular), the differences between the pO_2 of normal and abnormal skin were not significant (Fig. 6). Only the stasis ulcer bled easily on insertion of the electrodes. In all lesions, the degree of cellular infiltration was minor.

Discussion. Of the various factors controlling skin pO_2, several probably play only minor roles in tumor tissue oxygen tension. Little is known about oxygen diffusion coefficients and solubility in tissue, but it is very unlikely that any differences in these could be of a magnitude great enough to account for the observations reported. Oxygen use by the electrodes decreases as the measured pO_2 falls, and thus would affect readings in the opposite direction from that observed.

The two major determinants of tissue pO_2 are oxygen consumption and oxygen supply. The oxygen consumption of skin tumors has, (with exception of one observation(8)), been reported as increased over that of normal

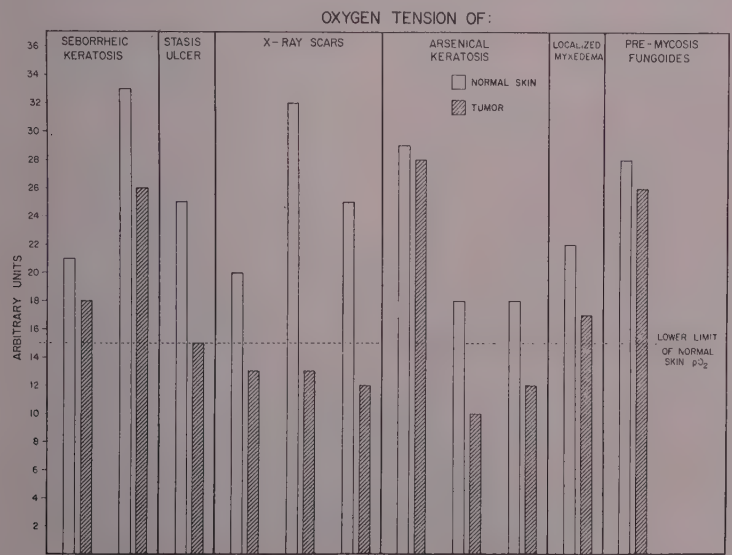

FIG. 6.

cutaneous tissue(9,10). The very low oxygen demand of normal skin(8,11) (QO_2 1.0-2.5) is thought to be due to the relatively small amount of respiring cell mass present, since more than two-thirds of the skin consists of metabolically inactive collagen and elastic fibres(11). Extensive replacement of such inert material by respiring cells (whether of malignant or of benign origin) could increase the oxygen consumption per unit volume of tissue and result in a drop in oxygen tension, provided blood flow remains the same.

Furthermore, malignant tumors are known to have a highly abnormal vascular system, characterized by lack of vessel differentiation, increase in volume of the vascular bed, and presence of very wide sinusoidal capillary channels(12-14). It is also probable that a significant amount of tumor blood bypasses the cell mass through arterio-venous anastomoses(13,15). This vascular anomaly probably results in a low blood flow (and reduced oxygen supply), resulting in decreased hemoglobin saturation and a steeper fall in oxygen tension within the capillaries. Hence the mean capillary oxygen tension (and similarly the tissue tension) will be lower. Experiments to be reported later demonstrate that tumor capillary blood in fact has a much lower mean pO_2 than that flowing in the capillaries of normal skin, which is probably near arterial.

As far as the non-malignant skin diseases examined, it has been shown by manometric methods that those lesions associated with inflammatory exudate or acanthosis have an increased aerobic respiration(16,17) while those lacking increase in cellularity have a normal or even reduced respiration(10). It has also been shown histochemically that, at least in psoriasis, the increased oxygen consumption is due to the increase in the number of epidermal cells and to the inflammatory cellular infiltrate(18).

It thus seems proper to conclude that the marked decrease of tissue oxygen tension observed in malignant cutaneous tumors is due to a combination of diminished capillary blood flow and a relative increase in the oxygen consumption of the diseased skin. In benign lesions characterized by acanthosis or cellular infiltration, but without the vascular anomalies of malignant tissue, the relative increase of oxygen consumption appears to be the major factor responsible for lowering tissue pO_2.

The relationship of our observation of a very low tumor tissue pO_2 to Warburg's theory of anoxic causation of carcinogenesis(19) is as yet unclear. If it could be demonstrated that local hypoxia precedes the development of malignant tumors, this hypothesis would be supported. Preliminary studies in our laboratories suggest that vascular changes leading to tissue hypoxia actually may precede chemical tumor induction in mice. However, until such experiments are completed and confirmed, judgment must be withheld.

One immediate application of our observations might be made in the field of radiobiology, since it has been shown that lowering of tissue oxygen tension protects against radiation injury(20). Thus the low tumor pO_2 would afford malignancies some measure of protection against therapeutic irradiation. If our observations can be extended to include noncutaneous malignancies, it appears reasonable that raising the low tissue oxygen tension might make some tumors more sensitive to the effects of radiation. Experiments to evaluate this procedure are now in progress.

Summary. 1. The tissue oxygen tension of a variety of benign and malignant cutaneous tumors was measured *in vivo* by means of a platinum oxygen cathode. 2. Malignant tumors, and benign lesions characterized by marked cellular infiltration or acanthosis, showed a very low oxygen tension as compared to that of the immediately adjacent normal skin. Benign lesions characterized by little or no increase in cellularity showed normal tissue pO_2. 3. The marked decrease of tissue oxygen tension of malignant tumors is probably due to a combination of an abnormality of the vascular system (insufficient oxygen supply) and a relative rise of oxygen consumption (resulting from a marked increase of respiring cell mass as compared to that of the normal skin). In benign cellular lesions, the latter factor is probably of primary importance. 4. The possible relation-

ships of our observations to the hypoxia theory of carcinogenesis and to tumor radiosensitivity are discussed.

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Intestinal Absorption of Unhydrolyzed Tripalmitin.* (22572)

RAYMOND REISER AND JULIUS W. DIECKERT.†

Department of Biochemistry and Nutrition, Texas Agricultural Experiment Station, Texas A. and M. College System, College Station.

One of the oldest and most important questions on the mechanism of fat absorption is the degree of absorption, if any, of unhydrolyzed triglycerides(1). Several years ago this laboratory published results of a study of absorption of C^{14} glycerol labeled conjugated trilinolein(2). Only about 60% of the labeled glycerol appeared in the lymph fat. It was concluded, therefore, that at least 40% of ingested triglycerides were completely hydrolyzed. The relative amounts of labeled glycerol in the saturated and unsaturated triglycerides of lymph fat after ingestion of mixtures of labeled fat with unlabeled saturated triglyceride, led to the conclusion that the remaining 60% of the ingested triglycerides were absorbed as monoglycerides.

The design of the above experiment was such that the absorption of relatively small amounts of unhydrolyzed triglycerides could not have been detected. The present study is an effort to determine more accurately if small amounts of unhydrolyzed triglycerides may be absorbed.

Methods. If a mixture of 10% saturated triglyceride and 90% unsaturated triglyceride are completely hydrolyzed and resynthesized by random distribution, the resultant fat will contain 0.1% saturated triglycerides. Any saturated triglycerides in the resultant fat above 0.1% will be equal to the quantity of

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† Present address: Oilseed Division, USDA Southern Utilization Research Branch, New Orleans, La.

the original which escapes hydrolysis. This principle was used to test the degree of absorption of unhydrolyzed tripalmitin by the measurement of labeled tripalmitin in thoracic duct chyle after ingestion of a mixture of 10% labeled tripalmitin, and 90% triunsaturated triglyceride. *Collection of lymph.* A 200 g white rat was fasted 24 hours and its thoracic duct cannulated at 2 p.m. After 10 hours the animal was offered one-half g of fat free synthetic ration plus a mixture of 20 mg of tripalmitin, C^{14} labeled in both glycerol and fatty acid moieties, and 180 mg of a triunsaturated glyceride.‡ The feed was promptly and completely consumed. The lymph became milky after about 20 minutes. Fifty ml of lymph was collected during the following 9-hour period, when it was quite clear. At 9:15 a.m. the rat was given a second meal containing 20 mg of doubly labeled tripalmitin and 180 mg of triunsaturated glyceride. The lymph again became cloudy in about 15 or 20 minutes. At 3 p.m., after lymph had become clear, lymph collection was discontinued. Approximately 50 ml were collected during the second period. The rat drank freely of 0.9% saline during lymph collection. *Isolation of saturated triglycerides.* The lymph collected during each period was diluted with alcohol, heated to denature the protein, and evaporated to dryness under reduced pressure. The dry residue was extracted with several small volumes of chloroform. The clear, dry chloroform solution was passed through a 10 g column of 100 mesh silicic acid to remove phospholipides(3). The chloroform solution of neutral fat and washings were pooled, evaporated to dryness, and weighed. During the first period 247 mg of fat were recovered from the lymph and 186 mg during the second period. The fat from each period was dissolved in 6 ml of acetone and the solution held at 25°C overnight. No crystals of saturated triglycerides appeared. It was probable that some saturated triglyceride was present but in too small quantity to be isolated by this method. Therefore, 125 mg of unlabeled tripalmitin was added as carrier and the solutions held at 8°C overnight and filtered(4). The precipitates were dissolved in another 6 ml of acetone and the crystallization repeated, but only for 2 hours. To wash out any unsaturated triglycerides containing labeled glycerol, 2 drops of triunsaturated triglycerides were added to the precipitates and the mixtures again crystallized from 6 ml of acetone. The crystals insoluble at 8°C were finally crystallized from 6 ml of acetone at 26°C overnight. The insoluble crystals were dried and weighed. From the first period lymph 122 mg of saturated triglycerides were obtained, and 119 mg from the second.

Results. Percentage of ingested palmitic acid recovered in the lymph. To estimate the percentage of ingested labeled palmitic acid which appeared in the lymph, the acetone soluble and insoluble fractions of lymph neutral fat of each period were saponified and the fatty acids weighed. The acids were dissolved in 10 ml of chloroform and volumes containing approximately 0.3 mg evaporated on planchettes and the specific activities determined. A similar determination was made of fatty acid of the labeled tripalmitin fed. Percentage of ingested labeled fatty acids which appeared in the lymph could then be calculated from the specific activities and weights of ingested and lymph acids. (Cts/min/mg x mg fatty acid = total cts/min. Total cts/min of lymph acids ÷ total cts/min ingested acids x 100 = percentage of ingested acids in the lymph.) The data are given in Table I.

Percentage of tripalmitin absorbed unhydrolyzed. Glycerol in saponification liquors of the lymph saturated triglycerides and ingested fat were oxidized with periodate and the resultant formaldehyde precipitated with dimethyldihydroresorcinol(2). The activity was corrected to infinite thinness. Since 4.8% and 3.8% of labeled fatty acids of lymph occurred in the saturated triglycerides, one might conclude, as a first approximation, that these quantities of labeled tripalmitin were absorbed unhydrolyzed. However, the ratio of specific activity of fatty acids to that of glycerol is 1.63 in the tripalmitin fed, but 2.23 and 2.13 in the saturated triglycerides of lymph fats of periods 1 and 2. This loss of

‡ Prepared from the unsaturated fatty acids of cottonseed oil.

TABLE I. Ingested Labeled Fatty Acids in the Lymph Fat.

Period	Ingested fatty acids			Lymph fatty acids						Lymph counts / Ingested counts × 100†
				Acetone soluble			Acetone insoluble			
	mg‡	C/m/mg*	Counts	mg	C/m/mg*	Counts	mg	C/m/mg*	Counts	% of total	Total counts	
1	17.7	9540	168858	308	393	120044	107	55.8	6026	4.8	126070	75
2	17.7	9540	168858	145	655	95975	109	34.2	3728	3.8	99703	59

* Counts/min./mg.
† These values are equal to percentages of ingested labeled fatty acids recovered from the lymph.
‡ Labeled palmitic acid from 20 mg of labeled tripalmitin.

active glycerol indicates some synthesis of lymph saturated triglycerides from hydrolyzed labeled fatty acids and unlabeled glycerol. For this reason the labeled glycerol in the saturated lymph triglycerides, and not the acids, should be used as a measure of triglyceride absorption.

From Table II it may be seen that the total glycerol activity ingested was 84215 counts per minute. In the first period 75% of ingested fatty acids appeared in the lymph. If the entire 75% had been absorbed as unhydrolyzed tripalmitin, the total glycerol activity of lymph saturated triglycerides would have been 63161 counts per minute. But only 2142 counts per minute or 3.3% of this theoretical amount was present. Therefore, 3.3% of the absorbed fat was unhydrolyzed.

In period 2, 3.2% of the absorbed fat was unhydrolyzed.

Discussion. The values of 3.3% and 3.2% of ingested tripalmitin which were concluded in the present study to have been absorbed unhydrolyzed, are the upper limits and probably too high. It has been demonstrated that about 60% of ingested fat is absorbed as monoglycerides(2). Resynthesis of these monoglycerides to triglycerides in the intestinal mucosa, in the present experiment, would result in about 0.06% labeled tripalmitin in lymph fat. The values of 3.3 and 3.2% are thus too high by at least 0.06%.

It is assumed in this and in earlier calculations(2) that if any triglyceride is absorbed, it does so without hydrolysis and resynthesis in the lumen of the intestine. Borgström has repeatedly(5-7) challenged this assumption. He has shown that in the lumen of the intestine as well as *in vitro,* pancreatic lipase is capable of resynthesizing triglycerides from monoglycerides and fatty acids. Theoretically, therefore, the assumption is false. Nevertheless, this factor can only invalidate the conclusions reached by the type of study herein described if the amount of rearranged triglycerides absorbed from the lumen is a significant fraction of the total. In Borgström's studies(6) 1.7 mg of labeled palmitic acid was fed in 1000 mg (roughly) of oil. Even at this very low ratio only 20% of free

TABLE II. Absorbed Unhydrolyzed Tripalmitin.

Period	Ingested tripalmitin glycerol* mg	C/m/mg	Cts/m	Lymph tripalmitin glycerol* mg†	C/m/mg	Cts/m	Cts/m‡	Absorbed unhydrolyzed tripalmitin, %
1	14.47	5820	84215	90.5	23.2	2100	63161	3.3
2	14.47	5820	84215	90.5	17.3	1566	49478	3.2

* As methylene bismethone.

† Calculated from the 125 mg of tripalmitin added to precipitate the traces of lymph tripalmitin.

‡ Theoretical value if all absorbed fat had been unhydrolyzed.

fatty acids were incorporated into intraluminary triglycerides. At the most (one molecule of fatty acid per molecule of fat) this could constitute only 0.1% of the intraluminary fat. At higher proportions of free fatty acids the percentage of incorporation should be expected to be even less. Borgström's studies are thus not designed to test the *degree* of randomization of intraluminary triglycerides. Other considerations, such as slow emptying time of fat from the stomach, rapid absorption of monoglycerides and free fatty acids and slow reaction time of lipase in the synthesis of triglycerides, are convincing arguments that the degree of such randomization must be low.

Summary and conclusion. A mixture of 20 mg of tripalmitin, C^{14} labeled in both the glycerol and acid moieties, and 180 mg of triunsatured triglycerides, was mixed in 1 g of a fat free ration and fed to a rat with a thoracic duct cannula. Lymph was collected for 9 hours. A second meal was fed and the lymph collected for 6 hours. It was found that in the 2 periods respectively, 3.3 and 3.2% as much of the labeled glycerol appeared in the lymph saturated triglyceride as would have appeared there had all the labeled lymph acid been absorbed unhydrolyzed. It was concluded, therefore, that only 3.3 and 3.2% of the tripalmitin was absorbed unhydrolyzed.

1. Reiser, R., *J. Am. Oil Chemists' Soc.*, 1954, v31, 292.

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3. Borgström, B., *Acta Physiol. Scand.*, 1952, v25, 101.

4. Reiser, R., and Dieckert, J. W., *J. Am. Oil Chemists' Soc.*, 1954, v31, 625.

5. Borgström, B., *Acta Physiol. Scand.*, 1952, v25, 328.

6. ———, *Arch. Biochem. and Biophys.*, 1954, v49, 628.

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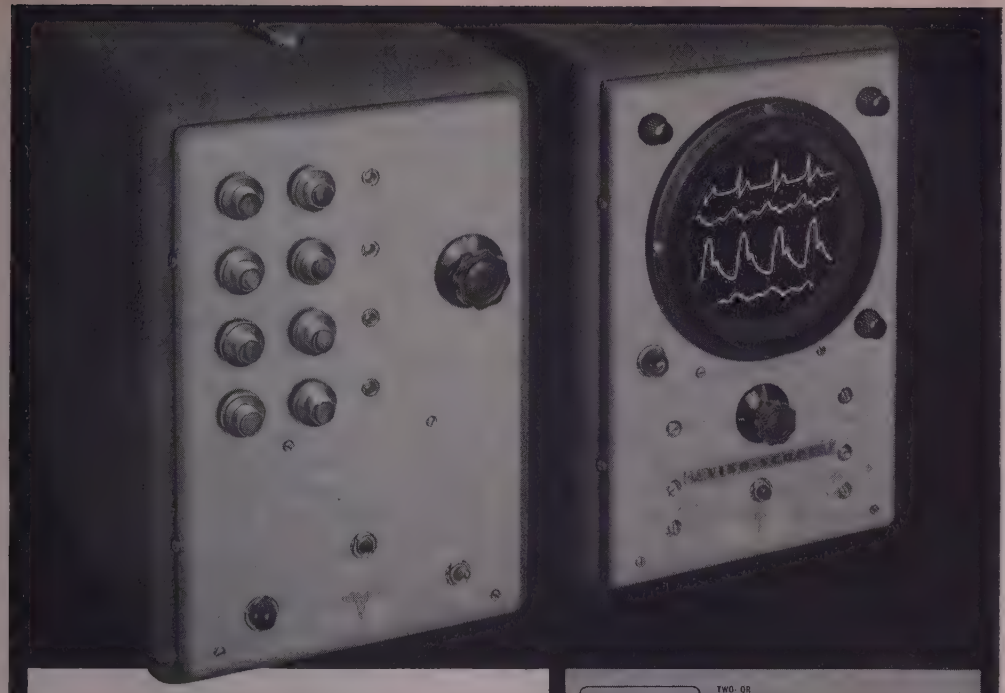
Received December 12, 1955. P.S.E.B.M., 1956, v91.



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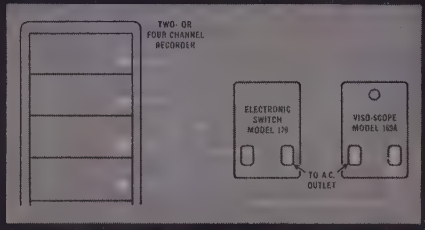

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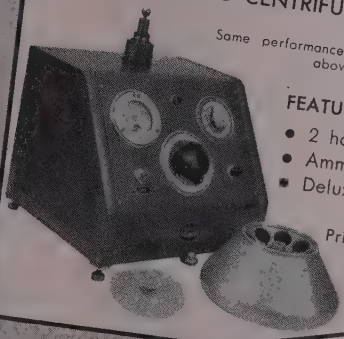

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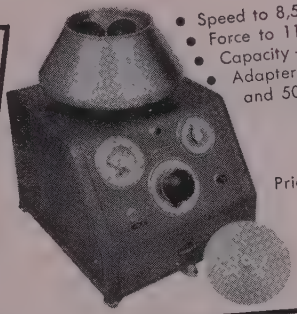

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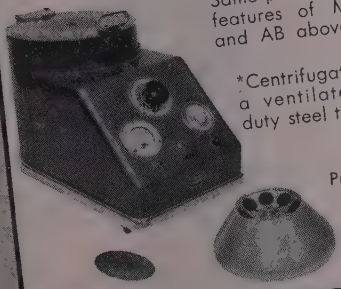

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1. J. Biol. Chem. *218*:335 (Jan.) 1956.

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2. Smith, H. W.: The Kidney, New York, Oxford University Press, 1951, p. 233.

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3. New York State J. Med. 55:489 (Feb. 15) 1955.

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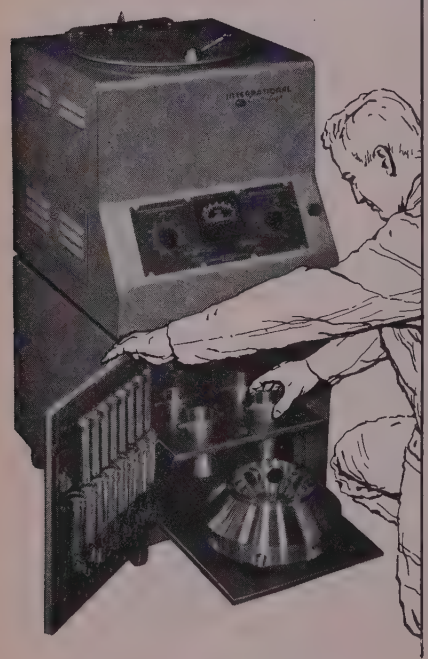

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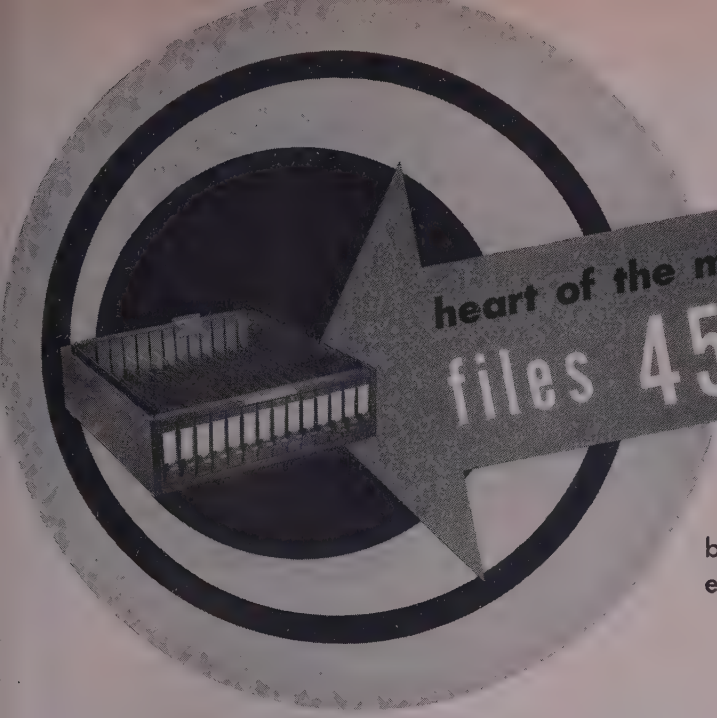

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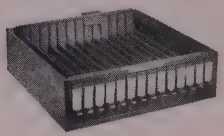

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